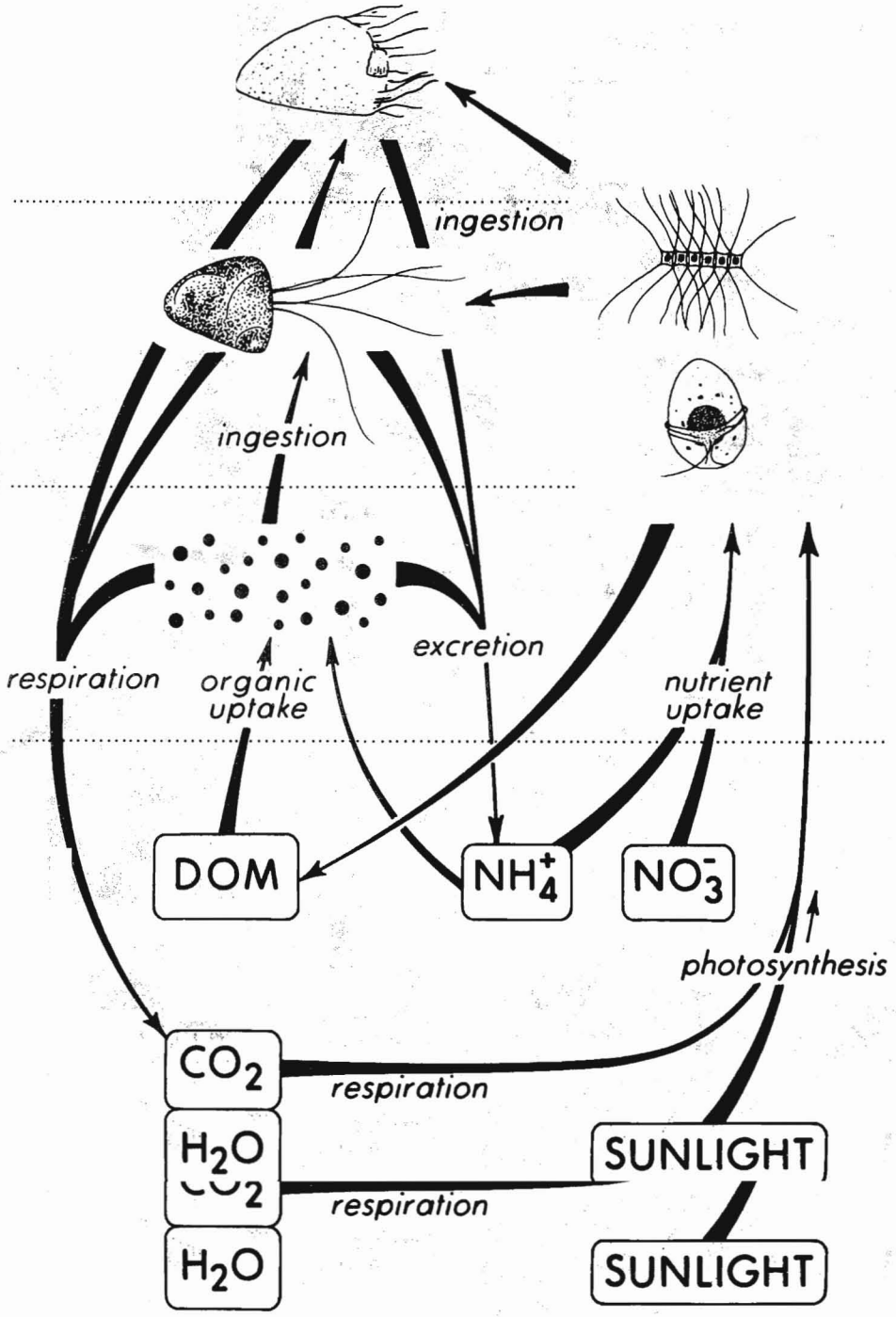
# NUTRIENT AND ENERGY CYCLING AMONG PHYTOPLANKTON, BACTERIA, AND ZOOPLANKTON





**EDWARD A. LAWS, EDITOR**



**1985 SUMMER PROGRAM**
**HAWAII INSTITUTE OF MARINE BIOLOGY**
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# NUTRIENT AND ENERGY CYCLING AMONG PHYTOPLANKTON, BACTERIA, AND ZOOPLANKTON

*Edited by*

Edward A. Laws

*Dedicated to the memory of*

Edwin W. Pauley

*Supported by a grant from the*

EDWIN W. PAULEY FOUNDATION

Hawaii Institute of Marine Biology

1985 Summer Study Program

Research Reports

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## DEDICATION

This volume is dedicated to the late Edwin W. Pauley, in recognition of the significant role that he played in the founding and development of the Hawaii Institute of Marine Biology on Coconut Island. In 1947, Mr. Pauley invited the University of Hawaii to establish a marine laboratory on Coconut Island (Moku o Loe) located in Kaneohe Bay, Oahu, Hawaii. This offer was accepted and Dr. Robert W. Hiatt was designated as the first Director of what was then known as the Hawaii Marine Laboratory. The original laboratory was housed in buildings donated by the Pauley family. Mr. and Mrs. Pauley took a very personal interest in the laboratory and they established close ties with the University community. This was especially apparent during the annual luncheon when they would invite the entire laboratory staff to their home on Coconut Island. Those who worked at the laboratory during this period have many fond memories of the Pauley's gracious hospitality at these gatherings. Mr. Pauley continued to be a good friend and provided considerable financial support to the laboratory over the years. He frequently visited the laboratory when he was in residence on Coconut Island in order to check on progress of various research projects and determine how he might help. When the original laboratory building was destroyed in a fire during 1959, he offered assistance and furnished funds to enable the University of Hawaii to begin construction of the present laboratory building which was completed in 1966. The laboratory continued to grow and prosper, until today it is one of the world's leading tropical marine research institutions.

At the marine laboratory we knew Mr. Pauley as a friend and as a person who had an abiding humanitarian interest in the advancement of science and in higher education. Mr. Pauley was, however, unique in many ways. He was a very successful businessman and recognized widely as an accomplished economist and diplomat. He had a distinguished career in government, education and private industry. He was a talented educator, a skilled educational administrator, an enlightened and judicious philanthropist and an enthusiastic promoter of arts and athletics.

Edwin Wendell Pauley was born in Indianapolis, Indiana, January 7, 1903, the son of the late Elbert L. and Ellen Van Patten Pauley. He graduated from Georgia Military Academy, College Park, Georgia, in 1918, enrolling the following year at Occidental College, Los Angeles, where he later served as a trustee.

After two years at Occidental, he transferred to the University of California at Berkley, receiving his Bachelor of Science degree from the College of Commerce and Business Administration in 1922.

His lifetime contributions in the field of education were enormous. Following graduation Mr. Pauley spent a year on the teaching staff at the University of California, Berkeley. His interest in education remained strong throughout his life, even though his primary occupation was in business and government service. He was appointed a Regent of the University of California in 1939. He was reappointed for a second sixteen-year term in 1954, and served as Chairman from 1956 to 1958 and from 1960 to 1962. He worked hard to shape the University of California into one of the world's greatest institutions of higher learning. In 1960-1961 Mr. Pauley played an active role in raising funds to establish KCET, Los Angeles' educational television station. He was awarded honorary degrees from the University of Santa Clara, and Pepperdine College, Los Angeles.

Mr. Pauley's principal business interest was the oil industry and in real estate development. After his teaching experience at the University of California, he became associated with his father in the Pauley Oil Company. In 1927, he organized the Petrol Corporation and operated it until 1948. He sold this company and subsequently engaged in the oil business under his own name. He did likewise in real estate and operated both businesses until the incorporation of the Pauley Petroleum Inc. in 1958. Mr. Pauley was involved in a variety of other business activities. He was a partner in the Los Angeles Rams Football Club, and a principal in the Riverside International Raceway and the Valley Music Theatre, Los Angeles. He was a director of the San Francisco - Oakland Television, Inc., which operated KTVU. In 1945, Mr Pauley purchased controlling interest in the Pacific Tire and Rubber Co. In 1951 he became interested in developing real estate in northern California and subsequently developed shopping centers and industrial parks in Southern California. The development of Hastings Ranch in Pasadena is an example. In addition, Mr. Pauley was a member of the Board of Directors of Western Airlines.

Mr. Pauley was an organizer and a leader in both business and government. In 1933, during the Great Depression, he represented the independent oil producers in the planning and coordinating committee under the National Recovery Act. From 1934 to 1938 he was president of the Independent Petroleum Association. He served as representative of the Governor of California at the Pan American High-

way Conference in 1939, and in the same year served as the Special Representative of the Governor of California on the National Resources Commission. With the approach of World War II, oil supply became vital to the defense of the United States. Mr. Pauley was appointed Special Representative of California on the Interstate Oil and Compact Commission in 1940 and was an organizer and member of the Defense Council of the State of California in 1941. In 1940 he was asked by President Franklin D. Roosevelt to serve as his Special Representative, acting as liaison between the United States and Great Britain. In 1941 he was also appointed by President Roosevelt to formulate plans for the Coordinator of Petroleum Industry, and later that year carried out missions in Europe as Special Representative of the Petroleum Coordinator for War. Mr. Pauley was economic advisor for the United States Government at the Potsdam Conference in 1945. From 1945 to 1946 he was the United States Representative on the Allied Commission on Repatriations, with the rank of Ambassador. From 1947 to 1948 he was Special Assistant to the Secretary of the Army.

In the Los Angeles community, Mr. Pauley's civic activities included the following: Director and Treasurer of the Hollywood Bowl Association, Member of the Board of Directors of the Los Angeles Chamber of Commerce, Member of the Board of Directors and President of the Los Angeles World Affairs Council, Member of the Southern California Committee for the Olympic Games, and Vice Chairman of the Board of Trustees of the California Museum Foundation. Mr. Pauley is one of the founders of the Los Angeles Music Center, which was established in 1964. He was an active supporter of the San Gabriel Valley Council of the Boy Scouts of America. He was a moving party and principal donor of land and new building which is the headquarters of the San Gabriel Valley Council. Mr. Pauley's other philanthropies included substantial contributions to the University of California.

Edwin Pauley died in July 1981 at the age of 78.

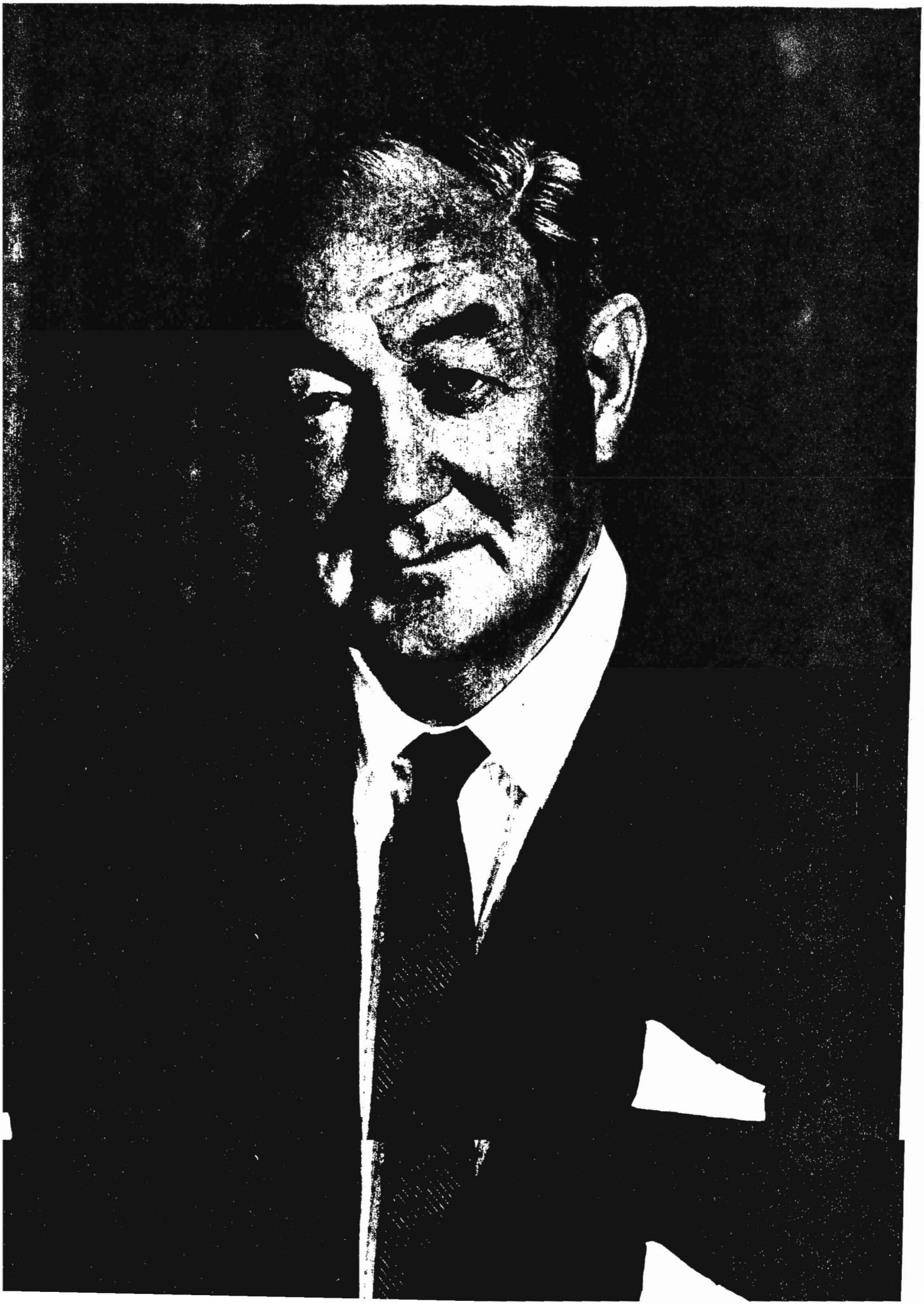
There is adequate evidence that Mr. Pauley was a man of great wisdom and vision who demonstrated a unique talent to identify areas of high potential success and to develop these areas. Perhaps it is not surprising that he recognized the importance of the marine sciences in the late 1940's some two decades before this area achieved national prominence. He had an abiding humanitarian concern for the potential role of marine science to address societal needs, particularly food production and resource management. Mr. Pauley correctly antici-

pated the important and unique role that the University of Hawaii was to play in the development of this area of research.

All of us at this laboratory recognize that Mr. Pauley gave up his privacy on Coconut Island when he invited the University to establish laboratories and share his beautiful estate. It is clear that he wanted this institution to grow and prosper. In this spirit, we have initiated a major summer research training program and have invited students and scientists from all over the world to participate.

The traditions started by Mr. Pauley have been perpetuated by his family and through the Edwin W. Pauley Foundation. Close ties have long existed between marine scientists from throughout the University of California system and HIMB due to joint programs initiated by Mr. Pauley. Mr. Pauley's wife, Barbara, takes an active, ongoing interest in the laboratory and has done much to promote marine science in Hawaii on her own initiative.

This volume represents only a tiny fraction of the research and training that has occurred at HIMB as a result of Edwin Pauley's foresight and generosity. The Hawaii Institute of Marine Biology is living evidence of Mr. Pauley's commitment to excellence in higher education and research in the marine environment. In a small way this dedication is a grateful recognition of our debt to this man.



Abstract

During the summer of 1985 students and senior faculty who participated in the HIMB Summer Studies Program utilized a variety of experimental techniques to study nutrient and energy cycling among phytoplankton, bacteria, and zooplankton. Bacterial production rates were found to vary by more than an order of magnitude between different locations in the bay and HIMB lagoon, with most of the production being due to free-living (not particle-bound) bacteria. Use of metabolic inhibitors indicated that bacteria played an important role in both the uptake and regeneration of phosphate, and that phytoplankton were by no means the only important consumers of phosphate. Fifty percent or more of phytoplankton photosynthetic rates was due to picoplankton (0.2-2 μm). The productivity/biomass ratio for the phytoplankton community was consistent with recent studies which have indicated that the cells are growing rapidly with little reduction in growth rate due to nutrient limitation. A new technique for estimating zooplankton grazing rates using fluorescent microspheres yielded results in excellent agreement with known grazing rates measured in a continuous culture system. Use of bacteria uniformly labeled with $^{14}C$ was found to be a promising technique for estimating bacteriovore grazing rates and assimilation efficiencies. Studies of the benthic diatom Nitzschia graeffei indicated that 6-40% of the carbon assimilated by this organism was obtained via photoheterotrophy.

## Introduction

The importance of nutrient and energy cycling among microorganisms in aquatic environments was stressed in a classic paper by Pomeroy (1974) over ten years ago, and has received increasing attention since that time as experimental results point more and more to the critical role played by aquatic microorganisms in assimilating and recycling both organic and inorganic substrates (Azam et al. 1983: Currie and Kalff 1984; Ducklow et al. 1986). In marine environments and particularly in oligotrophic areas the old paradigm of the diatom-copepod-fish food chain has given way to a food web model in which considerable cycling of substrates among microorganisms is required in order to explain experimental observations (Williams 1981). Perhaps the most imaginative such model in that of John Isaacs (1972, 1973) who used a so-called unstructured food web model to explain the distribution of trace elements among various organisms in a marine food web.

One of the major factors limiting the investigation of aquatic microbal ecology and the testing of hypotheses concerning the position and role of various microorganisms in the aquatic food web has been the lack of adequate methods for quantifying important rate processes. For example, how does one measure bacterial production? Fuhrman and Azam (1980, 1982) proposed a method for measuring heterotrophic bacterial production based on the assimilation of tritiated [methyl-$^{3}$H] thymidine. Present modifications of the method (Scavia et al. 1986) call for

measuring the incorporation of thymidine into DNA, and assuming a constant ratio between the production of new cells and the incorporation of thymidine. Karl (1982) has been critical of the thymidine method, and has argued in particular that the method is flawed because no allowance is made for the effects of intracellular pools and de novo synthesis on the specific activity of the thymidine within the cell. Karl (1981) proposed that the incorporation of tritiated [2-$^{3}$H] adenine be used as a measure of total microbial production. A key feature of the adenine method has been measurement of the intracellular ATP specific activity of the microorganisms under study. Fuhrman et al. (1986) have been critical of the adenine method, and have noted in particular that (a) there is no a priori reason to believe the intracellular ATP specific activities of all microorganisms within a population to be the same, and that (b) at least in some mixed populations there is evidence that bacteria greatly outcompete phytoplankton for dissolved adenine. As a result of (b), Fuhrman et al. (1986) argue that the adenine method is "inappropriate for measuring production rates in surface waters and other habitats where eucaryotic biomass dominates, such as sinking detritus." In response, Karl and Winn (1987) cite a variety of data which support their contention, "that adenine is assimilated by both algae and bacteria and that our adenine protocol measures total microbial production."

Although controversy surrounding relatively new methods such as the thymidine and adenine assimilation techniques is not unusual and could perhaps even be argued to be a healthy sign, in recent

years even old tried-and-true methods such as the $^{14}C$ technique for measuring primary production (Steemann Nielsen 1951) have been called into question (Peterson 1980). Two major concerns have been the possible adverse effects of confining phytoplankton in small bottles (Gieskes et al. 1979) and trace metal contamination (Fitzwater et al. 1982). Although systematic studies have revealed that neither bottle confinement problems (Sharp et al. 1980) nor trace metal effects (Marra and Heinemann 1983) are consistently or even commonly observed in $^{14}C$ incubations, it has nevertheless been argued that historical $^{14}C$ productivities are systematically low (Tijssen 1979; Postma and Rommetts 1979; Shulenberger and Reid 1981). These arguments have been based primarily on observed changes in oxygen ($O_2$) concentrations, which are concluded to imply photosynthetic rates from two to over ten times the historic $^{14}C$ values. In a series of papers Platt (1984), Platt et al. (1984), and Platt and Harrison (1985, 1986) have argued that this latter conclusion is unwarranted, and that when various considerations and sources of error are properly taken into account, there is no statistically significant difference between seasonal ($O_2$) accumulation data and $^{14}C$ production numbers in the open ocean.

It seems fair to say that at this time there is much controversy in the field of marine microbial ecology, and that this controversy stems in large part from either real or imagined inadequacies in the techniques used to study microorganisms. A quantitative understanding of the role of microorganisms in aquatic food webs has a variety of important practical

applications, including in particular fisheries management (Rhyther 1969) and estimation of pollution effects (Laws 1983). It is therefore of interest both from a practical and purely scientific standpoint to carefully examine potential problems with currently used methods and to hopefully develop new and better techniques for studying nutrient and energy cycling among these microorganisms. During the summer of 1985 a group of biological oceanographers and marine microbiologists conducted a twelve-week course entitled "Nutrient and Energy Cycling among Phytoplankton, Bacteria, and Zooplankton" at the Hawaii Institute of Marine Biology. This summer study program gave selected graduate and undergraduate students a chance to interact with some of the foremost marine microbial ecologists, to learn the latest techniques for studying marine microorganisms, and to conduct experiments using these methods and/or proposed new methods. A syllabus for the course was as follows:

SYLLABUS

| Dates | Topic | Lecturer | Institution |
|---|---|---|---|
| 5/28 - 6/02 | Orientation & Initiation of Field Work | | |
| 6/03 - 6/09 | Review of Current Concepts & Problems | Edward Laws | Univ. of Hawaii Honolulu, HI |
| 6/10 - 6/16 | Microbial Production - DNA Synthesis | David Karl | Univ. of Hawaii Honolulu, HI |
| 6/17 - 6/23 | Nutrient Cycling | William Harrison | Bedford Inst. of Oceanography Dartmouth, Nova Scotia, Canada |

Syllabus (continued)

| | | | |
|---|---|---|---|
| 6/24 - 6/30 | Algal - Bacterial Interactions | Satoru Taguchi | HI Instit. of Marine Biology Kaneohe, HI |
| 7/01 - 7/07 | Energy Budgets | Michael Mullin | Inst. of Marine Resources, Scripps Inst. of Oceanography La Jolla, CA |
| 7/08 - 7/14 | Bacterial Production- Thymidine Incorporation | Farooq Azam | Inst. of Marine Resources, Scripps Inst. of Oceangraphy La Jolla, CA |
| 7/15 - 7/21 | Vertical Transport of Biomass | Paul Bienfang | Oceanic Inst. Honolulu, HI |
| 7/15 - 7/21 | Enzyme Assays | Ian Morris | Univ. of Maryland Center for Environmental & Estuarine Studies Cambridge, MD |
| 7/22 - 7/28 | Zooplankton Grazing, Excretion, Respiration | Michael Pace | Univ. of Hawaii Honolulu, HI |
| 7/29 - 8/04 | Algal & Bacterial C and N Metabolism | Paul Falkowski | Brookhaven National Laboratory Upton, NY |
| 8/05 - 8/11 | Food Web Models | Pace/Laws | Univ. of Hawaii Honolulu, HI |
| 8/12 - 8/15 | Presentation of Results | Students | |

Students who participated in the program were:

| | |
|---|---|
| Megan Bailiff | University of Hawaii |
| Betsy Chronic | University of Alaska |
| Darrell Fox | University of Hawaii |
| Adrienne Gaedeke | University of Constance (Germany) |
| Paul Haberstroh | University of Washington |
| Margaret Murphy | Harvard University |
| Michelle Nawrocki | University of Hawaii |
| Cynthia Tynan | University of Rhode Island |
| Paul Zimba | University of Maryland |

The following reports summarize work carried out by these students. In some cases the experiments represented a group effort under the direction of one of the senior staff; in other cases the experiments were carried out by a particular student, but again with the counseling of one or more senior staff members. Although these experiments do not cover all possible aspects of bacterial-zooplankton-phytoplankton interactions, they did provide the students with an introduction to a broad range of techniques as well as a feeling for some of the problems and intriguing questions which arise in marine microbial studies.

Measurement of Bacterial Secondary Production by Thymidine Incorporation (directed by Farooq Azam; data analysis by Margaret S. Murphy).

## Introduction

Heterotrophic bacteria assimilate $^{3}$H-thymidine via a salvage pathway and convert it to dTMP using thymidine kinase. Seawater samples are incubated with [methyl-$^{3}$H]-thymidine to label the DNA synthesized during the incubation. A set of assumptions (Fuhrman and Azam 1982) is used to convert the rate of thymidine incorporation into a DNA synthesis rate. It is assumed for example that thymidine accounts for 25% on a molar basis of the nucleotide bases in DNA, and that the weight of the DNA in grams is equal to 1235 times the number of moles of thymidine in the DNA. The method is sensitive enough to measure significant incorporation within minutes to hours. [Methyl-$^{3}$H]-thymidine is used to minimize RNA labeling via $^{3}$H-uracil.

## Materials and Methods

Water samples were collected from five locations near HIMB (Fig. 1) using a clean, metal-free (2N HCl washed) sampler. Care was taken to avoid exposing the samples to bright light. The five locations included Kaneohe Bay (1), the lagoon next to HIMB (2), the saltwater pool adjacent to the Pauley estate (3), the Mahimahi aquaculture tank (4), and a saltwater aquarium (5). Quadruplicate 5-ml aliquots were pipetted into 17 x 100 mm, 13 ml capacity snap-top tubes. To one of these tubes was added formaldehyde at a final concentration of 2%. The formaldehyde

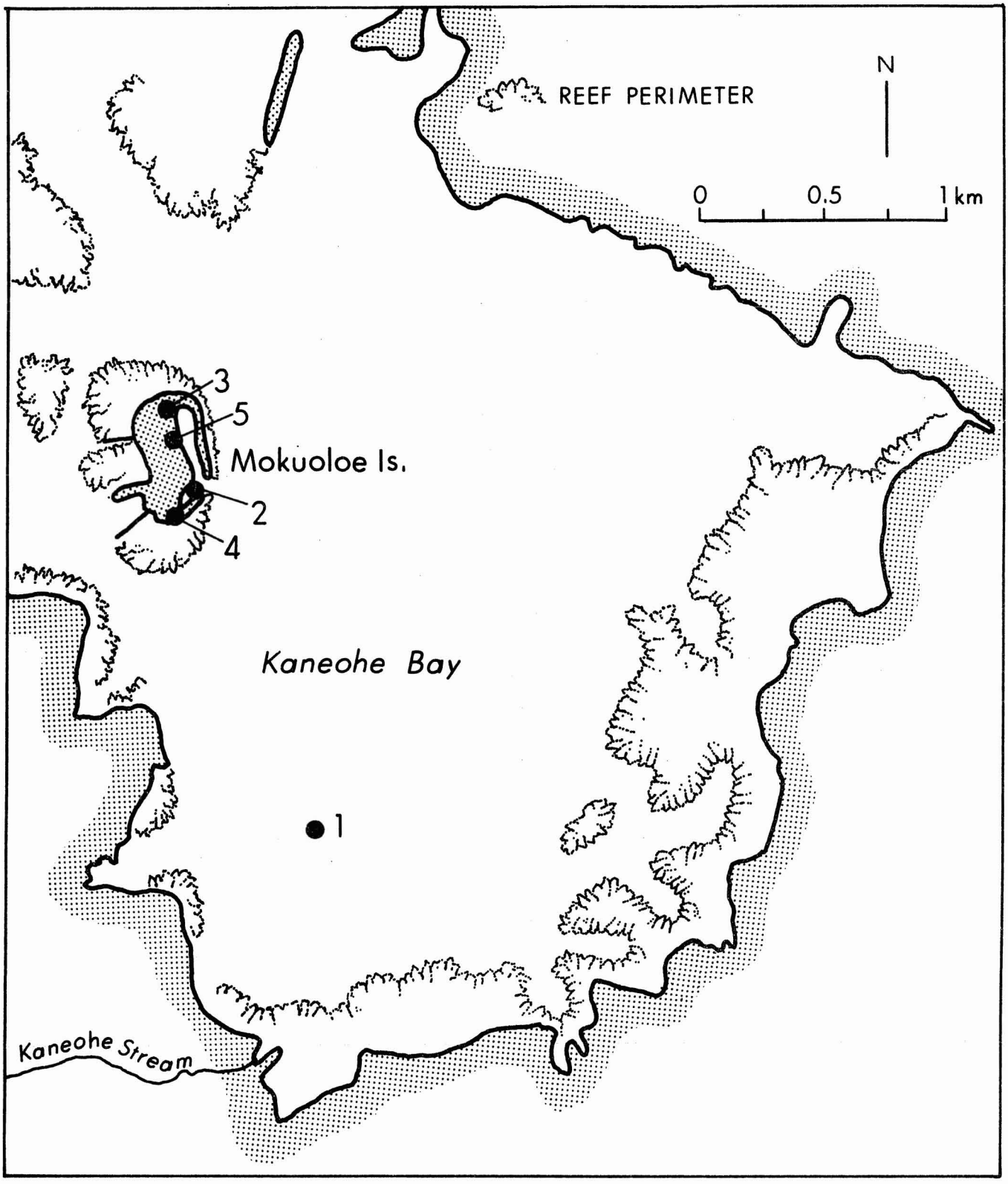



Fig. 1. Location of sampling stations in Kaneohe Bay. Station identifications are as follows: (1) Kaneohe Bay near abandoned sewer outfall, (2) the lagoon next to HIMB, (3) the saltwater pool adjacent to the Pauley estate, (4) the mahimahi aquaculture tank, and (5) a saltwater aquarium.

tube served as a control for the other three tubes. The tubes were placed in a rack in a shaded flowing seawater table, and the temperature allowed to equilibrate for 5 - 10 minutes. [Methyl $T^{-3}H$]-thymidine with a specific activity of 50 Ci/mM was then added to each tube at a final concentration of 10nM (ie. 5.55 x $10^6$ dpm per 5 ml tube). A 100 ml aliquot was taken from one of the tubes from each location to check the total activity. The samples were incubated for two hours, and then chilled in ice for 5 minutes. The contents of each tube was then filtered onto 0.45 µm (HAWP) Millipore filters with little or no vacuum.

Two ml of a 25% cold TCA solution were then passed through each filter, again with little or no vacuum. In a parallel experiment, either 1 ml or 5 ml samples from Kaneohe Bay and the saltwater pool were first filtered onto 1 µm Nuclepore filters to isolate particle-bound bacteria. The Nuclepore filters were then placed on 0.45 µm Millipore filters, and rinsed with two ml of cold 25% TCA as in the other experiments. Since cold TCA precipitates macromolecules (RNA, DNA, protein) almost instantaneously, it is acceptable to trap the particle-bound bacteria on the 1 µm Nuclepore filter and to then catch the precipitated macromolecules on the 0.45 µm Millipore filter immediately below the Nuclepore filter. Following the cold TCA rinse, the filters were transferred to scintillation vials, and one ml of ethyl acetate added to dissolve the filter. Finally ACS scintillation cocktail (Amersham) was added and the activity in the vials assayed on a Packard Tri-Carb model 4640 liquid scintillation counter.

## Results

The thymidine counts are shown in Table 1. If $5.55 \times 10^6$ dpm had in fact been added to each 5 ml tube, the expected activity in a 100 µl aliquot would be $1.11 \times 10^5$ dpm. It is apparent from Table 1 that the actual activity in the 100 µl aliquots consistently exceeded this value by about 20%. This observation immediately leads to some uncertainty in the calculated results, because it is unclear whether one should assume the specific activity of the thymidine to be 50 Ci/mM, as stated by the manufacturer, or about 20% higher than this figure. As a compromise, we assumed the specific activity to be the geometric mean of the value stated by the manufacturer and the value implied by the activity in the 100 µl aliquot. We illustrate the calculation procedure for the case of the Kaneohe Bay data.

$$\text{specific activity} = \frac{(1.347 \times 1.11)^{1/2}}{1.11 \times 10^5} \times \frac{10^5}{} \times 50 \text{ Ci/mM}$$

$$= 55 \text{ Ci/mM}$$

$$= 1.22 \times 10^{17} \text{ dpm/mole}$$

$$\text{moles DNA produced} = \frac{(17027 - 7791)}{1.22 \times 10^{17}} = 7.57 \times 10^{-14}$$

$$= 1.82 \times 10^{-10} \text{ moles } l^{-1} d^{-1}$$

$$= 224 \text{ pg DNA } l^{-1} d^{-1}$$

Table 1. Disintegrations per minute (DPM) in samples incubated for two hours with [methyl - $^{3}H$]-thymidine. Commas separate replicate counts.

| Sample Identification | DPM |
|---|---|
| **Kaneohe Bay** | |
| 100 µl aliquot from 5 ml | 134682 |
| blank | 2 |
| 5-ml sample | 18593, 19144, 13343 |
| 5 ml control (formaldehyde) | 7791 |
| **HIMB Lagoon** | |
| 100 µl aliquot from 5 ml | 137209 |
| blank | 2 |
| 5-ml sample | 81329, 90392, 94278 |
| 5-ml control (formaldehyde) | 9395 |
| **Saltwater Pool** | |
| 100 µl aliquot from 5 ml | 133297 |
| blank | 3 |
| 5-ml sample | 122720, 127272 |
| 5-ml control | 7393 |
| **Mahimahi Tank** | |
| 100 µl aliquot from 5 ml | 129273 |
| blank | 0 |
| 5-ml sample | 314402, 316603, 327759 |
| 5-ml control (formaldehyde) | 8437 |
| **Aquarium** | |
| 100 µl aliquot from 5 ml | 135783 |
| blank | 1 |
| 5-ml sample | 9954, 9785, 10764 |
| 5-ml control (formaldehyde) | 8475 |

Table 1. (continued)

Kaneohe Bay - test for particle-bound bacteria

| | |
|---|---|
| 100 µl aliquot from 5 ml | 118021 |
| 5-ml sample on 1 µm Nuclepore | 8653, 7509, 5717 |
| 5-ml control (formaldehyde)* | 7791 |
| 1-ml sample on 1 µm Nuclepore | 3253, 3932, 2826 |

Saltwater Pool - test for particle-bound bacteria

| | |
|---|---|
| 100 µl aliquot from 5 ml | 120913 |
| 5-ml sample on 1 µm Nuclepore | 22212, 19611, 19257 |
| 5-ml control (formaldehyde) | 7555 |
| 1-ml sample on 1 µm Nuclepore | 7751, 5724, 5536 |

* Assumed to be the same as sample collected on 0.45 µm Millipore.

Table 2 lists the similarly calculated DNA synthesis rates for each location. In the last column we have listed rates of heterotrophic bacterial carbon production estimated using an assumed carbon:DNA ratio of 50 (Mandelstam and McQuillen 1976).

Calculated heterotrophic production rates in the various locations we sampled varied by over two orders of magnitude, with the Mahimahi tank having the highest productivity and the aquarium the lowest. The experiment to determine the contribution of particle-bound bacteria to total heterotrophic production yielded completely equivocal results for the Kaneohe Bay sample. The results from the 5 ml sample implied that particle-bound bacteria accounted for none of the production, while the results from the 1 ml sample implied that particle-bound bacteria accounted for 100% of the production. The results in the case of saltwater pool were less equivocal. The 5 ml and 1 ml samples implied contributions of 11% and 21%, respectively, from particle-bound bacteria to total production.

## Discussion

We have only one number with which to compare our production estimates. Laws and Redalje (1982) have reported photosynthetic rates in the southeast sector of Kaneohe Bay to average 312 ± 46 mg C $m^{-2}$ $d^{-1}$ following diversion of sewage from the bay in May, 1978. Since the average depth of the southeast sector is about 10 m, this production rate corresponds to about 31 mg C $m^{-3}$ $d^{-1}$ = 31 µg C $l^{-1}d^{-1}$. Our calculated heterotrophic bacterial production rate of 1.1 µg C l-1 d-1 is only 3.6% of this figure,

Table 2. Estimated heterotrophic bacteria production rates based on the data in Table 1.

| Sample Location | Heterotrophic Bacterial Production | |
|---|---|---|
| | pg DNA $l^{-1}d^{-1}$ | µg C $l^{-1}$ $d^{-1}$ |
| Kaneohe Bay | 224 | 1.12 |
| HIMB Lagoon | 1905 | 95.3 |
| Saltwater Pool | 2869 | 143 |
| Mahimahi Tank | 7699 | 385 |
| Aquarium | 41 | 2.04 |
| Kaneohe Bay | | |
| particle bound bacteria | | |
| 5 ml sample | -14 | -0.7 |
| 1 ml sample | 230 | 1.15 |
| Saltwater Pool | | |
| particle bound bacteria | | |
| 5 ml sample | 328 | 16.4 |
| 1 ml sample | 610 | 30.5 |

a result which suggests that bacteria account for a very minor part of the particulate carbon production in that system. The very high rate of bacterial production in the Mahimahi tank undoubtedly reflects the use of dead fish as a source of food for the Mahimahi. The Mahimahi tank is also shaded by trees which provide organic inputs in the form of leaf litter and also shield the bacteria from UV light.

The high bacterial production rate in the Pauley saltwater pool is of some interest, as the pool is used for swimming by the Pauleys. The rather small tidal exchange for the pool is undoubtedly helpful to the maintenance of a relatively large crop of bacteria. Our results give no indication that the bacteria are in any way pathogenic to humans, but this point might be worth investigating, as it is unclear from where the bacteria are obtaining the dissolved organics which they require for nutrition.

The HIMB lagoon exhibited a production rate similar to that of the saltwater pool. The lagoon also has a restricted circulation, and the inputs of dissolved organics in that case may come in part from the resuspension of sediments by water exhaust ports. The aquarium exhibited a low bacterial production rate comparable to that of Kaneohe Bay, a not surprising result since the water which is circulated through the aquarium is drawn directly from the bay. Indications are that the bay is low in dissolved organic matter.

The results of the particle-bound bacteria experiments indicate several methodological problems. The equivocal nature of the Kaneohe Bay results directly reflects the high control counts (Table 1). In a system such as this one it would obviously be desirable to reduce these blank counts in some way. However, in both the Kaneohe Bay and saltwater pool experiments, the productivity of the particle-bound bacteria was judged to be higher if only one ml of water was filtered. There are several possible explanations for this observation. First, the higher calculated production when 1.0 ml is filtered may be due in part to the sample's "soaking", but not actually passing through the filter. Since any filter has a finite capacity for such "soaking", the more water that is passed through the filter, the smaller the effect of the "soaking." If "soaking" is a factor, then the assumption that the blank for the 1 ml sample is 20% of the blank for the 5 ml sample may be incorrect. This question could obviously be addressed by taking a 1 ml control and 1 ml total counts sample. Another possible artifact could be created by damage to cells as they pass through the filter. The edges of the pores in a Nuclepore filter may act almost like knife edges to a bacterial cell, and the larger flow of water in the case of the 5 ml sample may have resulted in the "shearing off" or dislodging of particle-bound bacteria that in a gentler flow might not have entered the filtrate. This problem can presumably be minimized by using very slow (gravity flow) filtration rates. Since little or no vacuum was used in our experiments, it seems likely that the "soaking" phenomenon was primarily responsible

for the differences between the 1.0 ml and 5.0 ml results. If so, then the 5.0 ml results are the more reliable, and we conclude that particle-bound bacteria were responsible for only a small fraction of the heterotrophic bacterial production at both the Kaneohe Bay and saltwater pool locations.

It seems appropriate at this point to review briefly the major potential problems with the thymidine technique for estimating bacterial production. These problems include (1) organisms other than heterotrophic bacteria assimilate thymidine, (2) not all heterotrophic bacteria assimilate thymidine (3) the specific activity of the added thymidine underestimates the effective specific activity of the DNA precursor pool, (4) use of cold TCA does not separate DNA from other compounds into which thymidine may have been incorporated, and (5) the C:DNA ratio in heterotrophic bacteria may be substantially smaller than 50, and perhaps closer to 5. With respect to point (1), Karl (1982) has cited a number of publications (Casselton and Stacey 1969; Fink and Fink 1962; Michrina and Deering 1980; Plaut and Sagan 1958; Sagan 1965; Scherbaum and Louderback 1960; Steffensen and Sheridan 1965; Stocking and Gifford 1959; Swinton and Hanawalt 1972) in which [$^{3}$H] thymidine has been reported to be incorporated into the the DNA of eucaryotic algae, protozoa, yeasts, fungi, and slime molds. These results seem hard to discount. Whether organisms other than heterotrophic bacteria assimilate significant percentages of the [$^{3}$H] thymidine in natural communities containing a variety of microorganisms is a legitimate question. Our Kaneohe Bay results suggest that

phytoplankton probably took up little of the [$^3$H] thymidine added to that sample, and size fractionation as well as autoradiographic studies reported by Fuhrman and Azam (1982) and Fuhrman et al. (1986) indicated domination of thymidine uptake by heterotrophic bacteria. It is noteworthy that Fuhrman et al.'s (1986) studies were conducted during spring bloom conditions when phytoplankton dominated both microbial biomass and production. Thus, although organisms other than heterotrophic bacteria clearly have the capability to take up thymidine, it is not clear that such assimilation always or even frequently causes serious problems in the interpretation of field data. Rivkin (1986) for example has reported uptake of tritiated thymidine by eucaryotic algae, but concluded that, "It is unlikely that the incorporation of $^3$H-TdR by algae generally precludes using $^3$H-TdR uptake to measure bacterial metabolism during short term incubations."

With respect to problem (2), Karl (1982) notes that autoradiographic studies by Fuhrman and Azam (1982) indicated that "only between 34 and 50% of the recognizable bacteria in coastal seawater assimilate [$^3$H] thymidine." Reference to Table 1 in Fuhrman and Azam (1982) shows that this statement is indeed correct, but that in the case of 50% assimilation, a mixture of 15 amino acids was also assimilated by only 50% of the bacteria, and that the same bacteria assimilated both the amino acids and thymidine. The conclusion reached by Fuhrman and Azam (1982) was that the remaining bacteria, while viable, were not actively growing, and hence that thymidine was assimilated by all actively growing bacteria. However, in a second experiment 84%

of the bacteria assimilated a mixture of amino acids and glucose, while only 37% assimilated thymidine. Thus there is an indication that 100% of actively growing bacteria do not always assimilate thymidine. However, it is possible that inactive bacteria might respond to and assimilate added amino acids and/or glucose, just as a plant which had been placed in the dark for several hours might respond to being placed in sunlight by assimilating $CO_2$. In other words, the fact that a bacterium assimilates added amino acids and/or glucose does not necessarily imply that the organism was actively growing before the substrates were added.

The fact that the specific activity of the added thymidine may seriously underestimate the effective specific activity of the DNA precursor pool (problem 3) seems to be a cause for real concern. Fuhrman and Azam (1982) indicate that uptake rates are insensitive to amounts of added thymidine at concentrations in excess of 5 nM. Therefore the extracellular thymidine concentration is apparently small compared to 5 nM. However, serious dilution of the thymidine specific activity can apparently result from the combined effects of de novo synthesis and intracellular thymidine. Fuhrman and Azam (1982) used radioactive phosphorus as well as [$^3$H] thymidine to estimate DNA synthesis rates in nearshore and offshore microbial populations in the Southern California Bight. After incubations lasting 0.9 - 5h, the water samples were filtered through 0.6 or 1.0 $\mu$m filters to presumably remove all organisms except for bacteria, and the TCA-insoluble material in the filtrates assayed for

radioisotope incorporation. It was assumed that the effective specific activity of the phosphate could be accurately estimated from the ratio of the added amount of radioisotope and the measured phosphate concentration. Intracellular phosphate concentrations were assumed to be low and to rapidly equilibrate with the extracellular pool. Synthesis rates calculated from phosphorus uptake were 2.7 - 7.1 times rates estimated from the thymidine uptake. The implication was that the effective thymidine specific activity was in fact 2.7 - 7.1 times smaller than the specific activity of the added thymidine. Fuhrman and Azam (1982) concluded, "If realistic estimates of DNA synthesis are judged to be more important than conservative ones, the estimated rates of DNA synthesis should be increased by approximately a factor of 3 to 6 for nearshore and 6 to 7 for offshore waters." The magnitude and variability of this correction factor are both causes for concern, particularly when one considers that the figures are based on data from only three locations.

The fact that cold TCA does not separate DNA from protein and RNA, both of which can become labeled with thymidine (problem 4), is another concern acknowledged by users of the thymidine method. Although chemical separation of the DNA is certainly feasible, total TCA-insoluble incorporation is much easier to measure than incorporation into a purified DNA fraction, and can be done completely at sea with rapidity and ease, (Fuhrman and Azam 1982). Although there is agreement that both RNA and protein can be labeled with $^{3}H$ from [$^{3}H$] thymidine, the magnitude of the

correction factor to allow for this effect is unclear. Fuhrman and Azam (1982) concluded, "For the offshore environment, conservative estimates of growth would be provided by assuming that 65% of the TCA-insoluble label is DNA, but more realistic estimates of growth would use values of about 80%." However, in a study in Lake Michigan, Scavia et al. (1986) reported, "40-60% (mean = 47%) of the thymidine was incorporated into DNA," and studies by Karl (1982) have shown that the percentage incorporated into DNA may be as low as 30 - 35%. Thus there is about a factor of two uncertainty in the calculated result depending on what percentage of the acid-insoluble activity one believes to be confined to the DNA.

Finally we come to the question of the C:DNA ratio in bacteria. In fact many users of the thymidine method prefer to report results in terms of bacterial cells produced, the rationale being, "the DNA content per cell would be more constant than the DNA per unit weight" (Fuhrman and Azam 1982). However, at least in eucaryotic algal cells, Holm-Hansen (1969) has shown that DNA per cell can vary by at least a factor of 2000, and is highly correlated with the cell's carbon content. In fact, while DNA per cell varied by a factor of 2000, the C:DNA ratio varied by a factor of only three. Thus there is some basis for expecting that C:DNA ratios in bacteria would be more constant than DNA per cell, and in any case a knowledge of heterotrophic bacterial production in terms of carbon facilitates comparison with reported photosynthetic rates. Karl et al. (1984), Karl and Knauer (1984), Winn and Karl (1984), Burns et al. (1984), and

Craven and Karl (1984) have routinely assumed a C:DNA ratio of 50 based on the work of Holm-Hansen (1969), but the latter admittedly includes data on phytoplankton only. Interestingly, the C:DNA ratio in Holm-Hansen's cultures is negatively correlated with cell carbon content, and increases from a value of 30 at 6000 pg C/cell to 100 at 10 pg C/cell. Fuhrman and Azam (1982) argue that typical marine heterotrophic bacteria contain only about 1.7 fg C/cell. They then point out that if the C:DNA ratio in such bacteria were even as small as 30, the cells would contain only about 57 ag DNA, an amount which they consider much too small to meet, "the genetic requirements of an independently living organism." Based on DNA measurements and bacterial counts in the 0.2 - 0.6 μm size fraction of freshly collected seawater, Fuhrman and Azam (1982) conclude that the C:DNA ratio in marine bacteria is approximately 5. However, the calculation of this ratio required them to assume all the DNA in that size fraction to be associated with living cells, a dubious assumption considering the recent report by Winn and Karl (1986), which showed that only 10 - 25% of particulate DNA was associated with growing microbial cells at a series of stations in the North Pacific. Since it is the C:DNA ratio in marine bacteria rather than marine phytoplankton that seems to be in dispute, one partial resolution of this controversy would be to simply report heterotrophic bacterial production in terms of DNA synthesis, and for comparison purposes divide phytoplankton production in terms of carbon by the C:DNA ratio in phytoplankton.

In summarizing the various uncertainties in the thymidine

assimilation method, we note that correction factors of 3 - 7 must be applied to correct for dilution of the thymidine specific activity, correction factors of 0.3 - 0.8 must be applied to allow for the fact that not all the activity in the cold TCA fraction is confined to DNA, and that a factor of 5 - 50 must be used to convert DNA production to carbon production. The product of these three factors therefore could conceivably range between 4.5 and 280. The factor used in the calculations in Table 2 was 50, which is close to the geometric mean (35.5) of the range of conversion factors. Obviously the over sixty-fold uncertainty (4.5 to 280) in the conversion factor can be reduced by a factor of 10 if one is willing to report production in terms of DNA rather than carbon. Furthermore, one can certainly isolate the DNA from the RNA and protein (Riemann amd Sondergaard 1984), and thereby reduce the uncertainty by another factor of 2.7. One is then left with the uncertainty in the specific activity of the thymidine. This point would seem to deserve more attention if the thymidine method is going to be widely used, as a glance at the recent literature would seem to indicate. The factor of 3 - 7 is based on studies at only three stations by Fuhrman and Azam (1982), and the paucity of data leaves considerable uncertainty as to just how representative these correction factors are. For example, is the correction factor of three based on work at the Scripps Pier really representative of all coastal environments? We have no idea. Some additional work involving phosphorus and thymidine incorporation into DNA would be useful, as well as some studies with chemostats or batch cultures where the true rate of

DNA synthesis is known with a high degree of accuracy.

Measurement of Microbial Phosphate Uptake With the Use of $^{33}P$ (directed by William G. Harrison; data analysis by Margaret S. Murphy).

## Introduction

It has been recognized for many years that the supply of inorganic nutrients, and particularly nitrogen and phosphorus, is most likely the most important factor limiting the production of organic carbon over the great majority of the ocean's surface area (Redfield et al. 1963). Although experimental work has generally implicated nitrogen rather than phosphorus as the primary limiting nutrient in most parts of the ocean (Thomas 1970 a,b; Ryther and Dunstan 1971), some studies (Myers and Iverson 1981; Berland et al. 1980) have shown clear evidence for phosphorus limitation as well. Schindler (1977) has argued that the presence of nitrogen-fixing bacteria usually causes freshwater lakes to become phosphorus limited, and Smith (1984) has expanded on this argument to explain the relative importance of N and P limitation in both freshwater and marine systems. Smith (1984) argues that biologically mediated processes such as nitrogen fixation and denitrification can, given sufficient time, lead to either nitrogen limitation or phosphorus limitation, but that physical processes such as water exchange and mixing can effectively obscure such biological effects. Thus whether a given body of water tends to be N or P limited will depend both on the nature of the biological and physical processes at work and on their relative time scales. For example, deep ocean water

with a residence time of hundreds of years tends to be N limited when it rises to the surface because there has been ample time for denitrification to reduce the concentration of available N in the water. However, if such water remains at the surface and in a confined environment for sufficient time, nitrogen fixation may create a P-limited condition (Smith and Atkinson 1984).

The importance of N and P in controlling the rates of microbial processes in the ocean has led to the development of a variety of techniques for studying these processes. Use of isotope tracers has played an important role in the development of these methods. Unfortunately there is no suitably long-lived radioisotope of N ($^{13}N$, the longest-lived radioisotope of N, has a half-life of only 10m), and most studies of N cycling have therefore used the stable isotope $^{15}N$ (natural abundance 0.365%). Fortunately, P has two radioisotopes with reasonable half-lives, $^{32}P$ and $^{33}P$. Because of the availability of these radioisotopes, we chose to study P cycling as part of the 1985 HIMB Summer Studies Program. $^{33}P$, the longest-lived of the P radioisotopes (half-life = 25d), also fortuitously has the lowest decay energy, and hence is the safest for routine use. Our experiments were therefore conducted with $^{33}P$.

Several important questions have been raised recently with respect to the sort of studies we envisioned. First, it has more-or-less routinely been assumed that phytoplankton or macrophytes were almost entirely responsible for the uptake of both inorganic N and phosphate in aquatic systems. The

conventional wisdom on this point has been well summarized by Riley and Chester (1971, p. 155), who commented, "Phytoplankton normally synthesize their proteins from nitrate, nitrite and ammonia, but bacteria usually use these forms of nitrogen when organic nitrogen is not available." This point of view has recently been challenged by Harrison et al. (1985), Laws and Harrison (1985), Wheeler and Kirchman (1986), and Currie and Kalff (1984 a, b). Harrison et al. (1977) used size fractionation, metabolic inhibitors, and various organic and inorganic tracers. Their results showed that at least 50% of the phosphate uptake but only 10% of the chlorophyll were associated with the <1 μm fraction. Laws et al. (1985) compared phytoplankton N assimilation estimated from $CO_2$ incorporation into protein with ammonium uptake using $^{15}N$, and concluded that bacteria accounted for 50 - 75% of the ammonium uptake. Wheeler and Kirchman (1986) used size fractionation and specific inhibitors of protein synthesis, and concluded that ammonium accounted for at least 20 - 60% of the summed ammonium plus amino acid utilization by bacteria. Currie and Kalff (1984a) utilized size fractionation and various organic and inorganic tracers. They concluded that "bacterioplankton apparently strongly dominates orthophosphate cycling in situ, while the phytoplankton seems to obtain most of its phosphorus from excreted organic P compounds." Thus the intuitively appealing idea that heterotrophic bacteria obtain most of their N and P from dissolved organics may be misleading. Our experiments were designed in part to examine this question in the case of P.

A second question or problem which has arisen in recent studies of nutrient cycling concerns the method of data analysis. Early work with isotope tracers (Dugdale and Goering 1967) involved simply monitoring the transfer of tracer from the dissolved to particulate phases. Such measurements yielded estimates of uptake only. Later Blackburn (1979) and Caperon et al. (1979) noted that monitoring both the isotope ratio and concentration of the dissolved phase could yield estimates of both uptake and regeneration rates. Glibert et al. (1982) then noted that the dilution of tracer isotope concentration in the dissolved phase due to regeneration, a phenomenon explicitly utilized in the Blackburn-Caperon approach, should also be taken into account when calculating uptake rates utilizing the Dugdale and Goering (1967) procedure. One methodological problem has been the fact that ambient substrate concentrations of inorganic N and P are sometimes bordering on the limit of detection, and this fact can create considerable uncertainty in the calculation of rate constants. Glibert et al. (1982) devised a method of analysis for N uptake rates which avoids use of substrate concentrations, but requires that one assume a particular functional form of the substrate isotope ratio. Laws (1984) has shown that this particular functional form applies only when uptake and regeneration are exactly in balance or when the regeneration rate is zero. In the case of $^{15}N$ work the substrate ratio is measured on a mass spectrometer, but in $^{33}P$ work the isotope ratio must be calculated from a knowledge of the substrate activity and concentration. Therefore, there is no circumventing the need

for accurate substrate concentrations in $^{33}P$ work if one is to calculate either uptake or regeneration rates. This fact will become apparent in our data analysis.

## Materials and Methods

Surface water samples were collected on 6/20/85 from the southeast sector of Kaneohe Bay (Fig. 1) using a plastic bucket. The water was immediately dispensed into two-liter polycarbonate bottles, which served as incubation containers. Each bottle was inoculated with 4 $\mu$Ci carrier-free $H_3{}^{33}PO_4$ with an activity of approximately 21 $\mu$Ci/ml. One bottle served as a control and received no further additions. A second bottle was inoculated with cycloheximide, which inhibits polypeptide chain elongation in eukaryotic but not prokaryotic organisms (Dixon and Webb 1979). A third bottle was inoculated with chloramphenicol, which is known to inhibit peptidyl transferase of large sub-units, thus preventing formation of peptide bonds. Chloramphenicol acts only on bacteria, mitochondria, and chloroplasts (Dixon and Webb 1979). Both cycloheximide and chloramphenicol were added at concentrations of 100 $\mu$M. The incubations were conducted in outdoor water tables at HIMB beginning at approximately 9:50 A.M. Temperature control was provided by circulating seawater.

The incubation bottles were sampled at time zero (9:50 A.M.) and at 1, 3, 6, 12, and 24h after time zero. At each sampling time two 100 ml aliquots of water were taken from the bottles and filtered separately onto 0.22 $\mu$m Millipore membrane filters. The filters were rinsed with 10 ml of seawater, the filtration

chimney removed, and the circumference of the filter rinsed with a few ml filtered seawater while vacuum continued to be applied. The filters were then placed in scintillation vials for subsequent counting. The sample filtrates were combined, and 10 ml of pre-mixed phosphate reagents (ammonium molybdate, ascorbic acid, potassium antimonyl-tartrate, and sulfuric acid) added to bring about color development (Strickland and Parsons 1972). Ten minutes were allowed for the color to develop and stabilize. Twenty ml of isobutanol were then added, and the mixture shaken in a separatory funnel. The mixture was then allowed to stand for five minutes while the aqueous and alcohol phases separated, and the aqueous layer then drained off. The alcohol was then collected in a graduated cylinder, and the volume brought up to exactly 20 ml with ethanol. Two one-ml aliquots were then taken for liquid scintillation counting, and the remainder was analyzed for phosphate concentration on a Beckman model 35 spectrophotometer (Strickland and Parsons 1972). Scintillation counting utilized ACS scintillation cocktail (Amersham). The counting was done on a Packard Tri-Carb model 4640 liquid scintillation counter.

## Results

We were initially concerned to see whether the sum of the activities in the phosphate and particulate material remained constant. It is conceivable for example that some $^{33}P$ would be adsorbed to the sides of the incubation flask or be combined in a chemical form which was not efficiently isolated by filtering or

alcohol extraction. Fig. 2 shows the total activity in each of the three treatments as a function of time during the experiment. The average activity was $3.42 \pm 0.53 \times 10^6$ dpm/l. There is no evidence of a temporal trend in the total activity, and with the exception of the zero time activity the average values span a rather narrow range from 3.15 to $3.5 \times 10^6$ dpm/l. In analyzying the data, we have therefore assumed that the total activity was constant, or in other words that all P cycling involved exchanges between phosphate and the particulate phosphorus pool.

In analyzing P tracer experiments such as this one, it is customary to assume that the regenerated phosphate contains no $^{33}P$ (Harrison 1983). Hence regeneration causes the specific activity of the phosphate pool to decline with time. Even if some $^{33}P$ did begin to find its way into the regenerated phosphate, it is highly improbable that the specific activity of the regenerated phosphate would exceed that of the ambient phosphate. Thus anything other than a steady decrease in the phosphate specific activity with increasing time is to be viewed with great skepticism. Fig. 3 shows that the activity of the phosphate did in fact steadily decline with time, a trend of course reflecting the uptake of $^{33}P$. However, Table 3 shows that the specific activity of the phosphate did not steadily decrease with time. The problem can be traced directly to Fig. 4, which shows the measured concentrations of phosphate. Since only one analysis was done at each point in time, it is difficult to say what the precision of the numbers may be. Strickland and Parsons (1972) estimate that within 95% confidence the true value lies

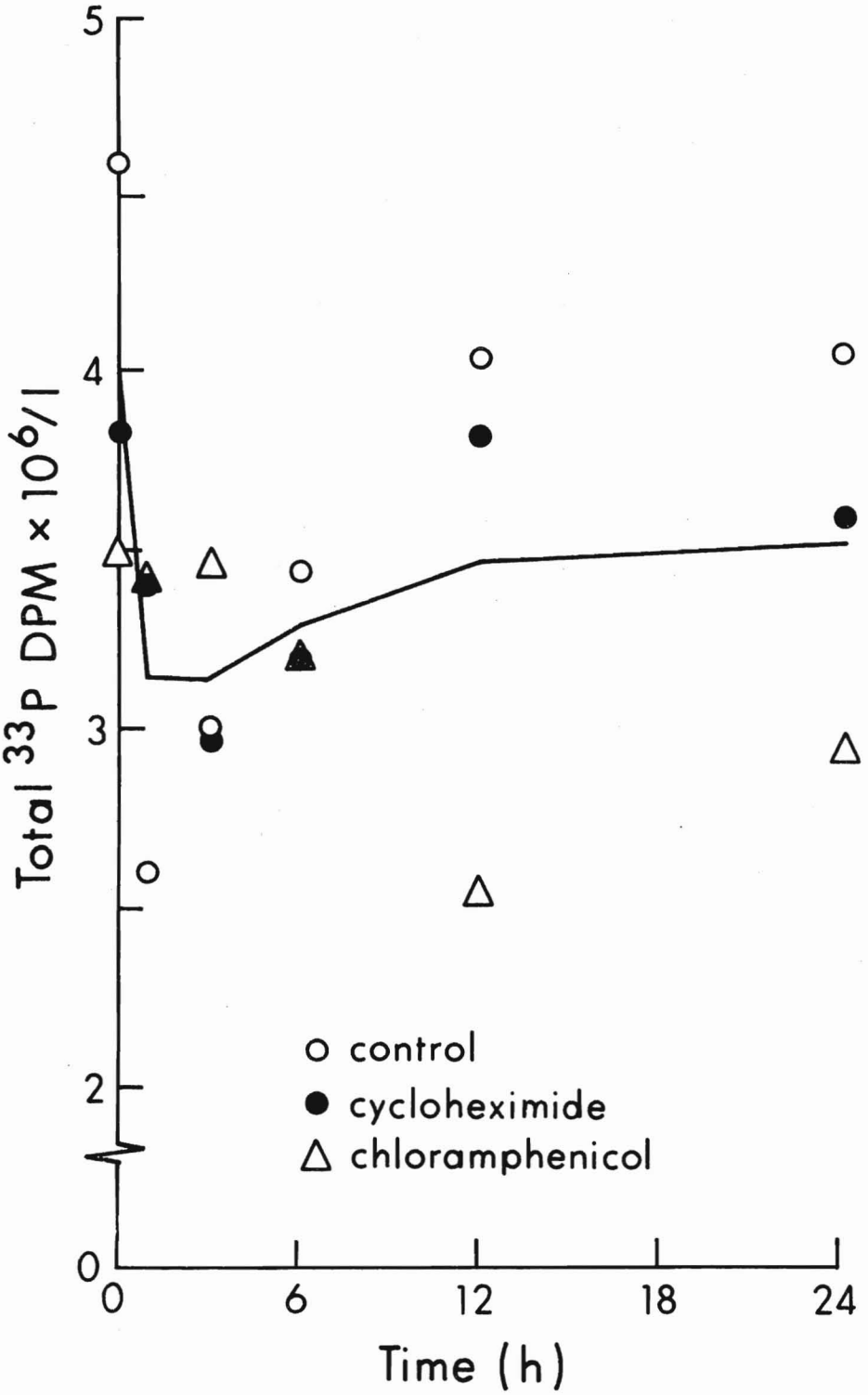
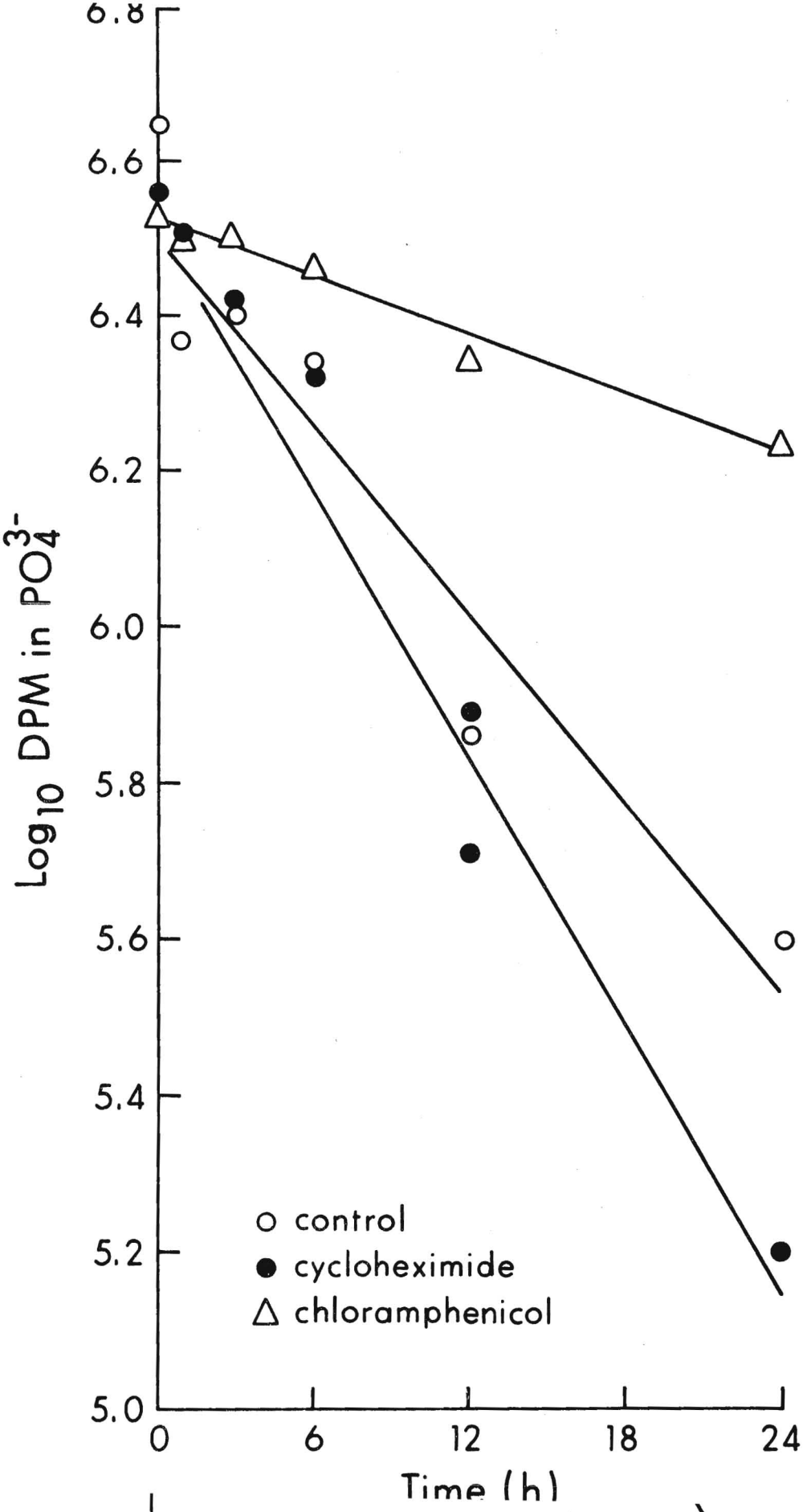



Fig. 2. Total $^{33}$P activity (dissolved plus particulate) in control flask and flasks treated with cycloheximide and chloramphenicol versus time.

0 6 12 18 24
Time (h)

Fig. 2. Total $^{33}$P activity (dissolved plus particulate) in


6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
Log10 DPM in PO4 3-
control
cycloheximide
chloramphenicol
0
6
12
18
24
Time (h)


5.2
control
cycloheximide
chloramphenicol

Table 3. Specific activity of phosphate as a function of time. Units are $10^6$ dpm/ $\mu$M.

| Time | Control | Cycloheximide | Chloramphenicol |
|---|---|---|---|
| 0 | 115 | 51.9 | 53.5 |
| 1 | 16.6 | 13.0 | 30.2 |
| 3 | 183 | 220 | 153 |
| 6 | * | 62.1 | 126 |
| 12 | 15.3 | 5.9 | 91.5 |
| 24 | * | 3.4 | 94.5 |

* Phosphate was analytically undetectable.

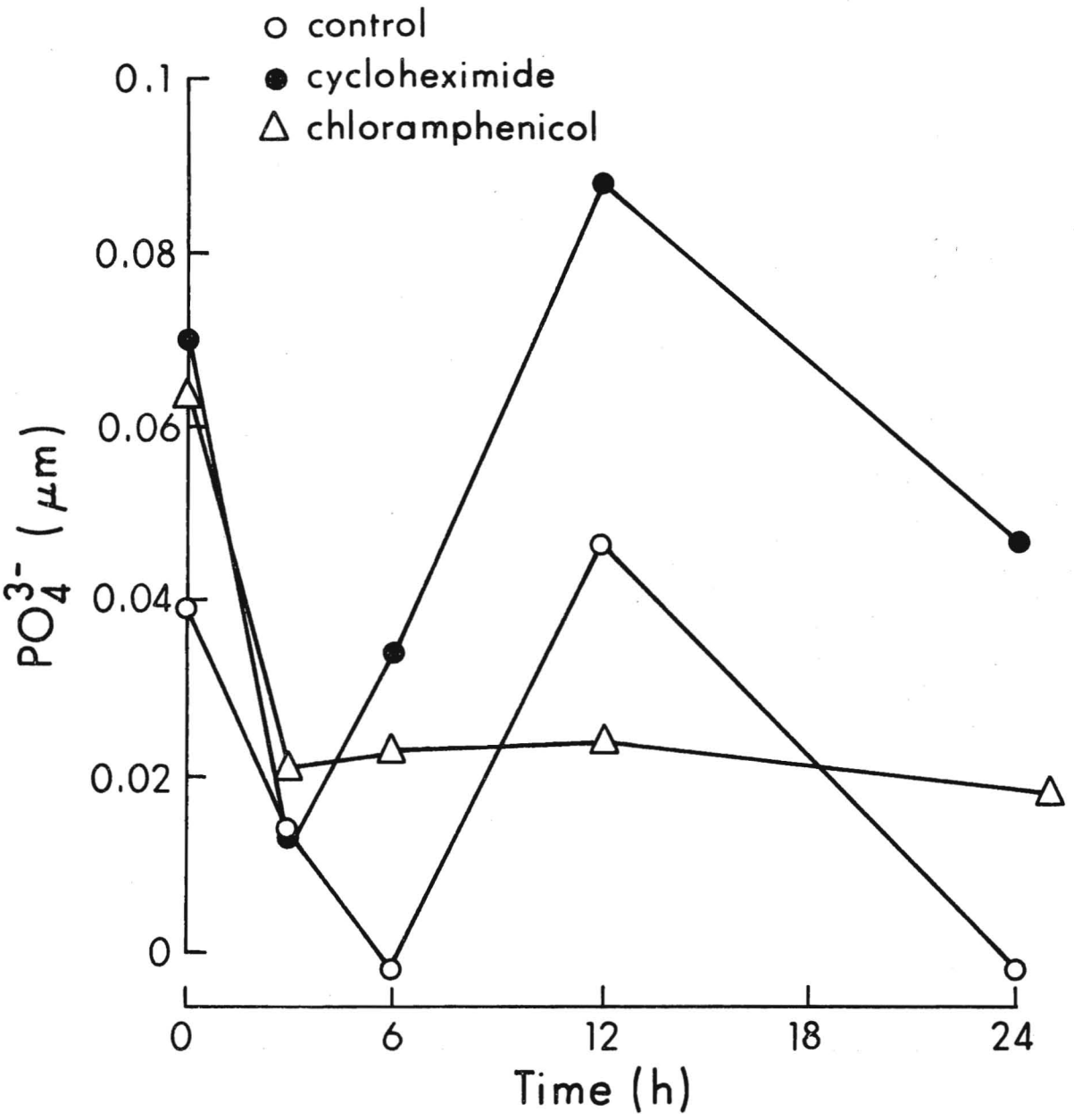
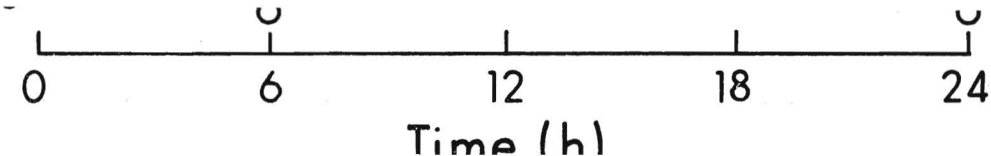



Fig. 4. Phosphate concentrations versus time in experimental flasks. Symbols as in Fig. 3.



within ±0.006 μM of the measured value if only one measurement is made, but that error estimate refers to replicate analyses on the same water sample. It is likely that the error bounds would be increased if analyses were made on separate water samples. In any case it is obvious from Fig. 4 that a change of even 0.006μM would have a significant effect on most of the measured phosphate values in the control and chloramphenicol experiments. Because the ambient phosphate values were so low and because there is no consistent temporal trend in any of the phosphate data, we elected to assume that the phosphate concentrations were constant in each case. We allowed three hours for the cycloheximide and chloramphenicol to take full effect, and therefore compared results only from the third to the twenty-fourth hour. The integral average phosphate concentrations over that time period were 0.021, 0.059, and 0.022 M in the control, cycloheximide, and chloramphenicol bottles, respectively. If the phosphate concentration is indeed constant, then the activity of $^{33}P$ in the phosphate should be described by the equation

$$A_t = A_o e^{-UT/S} \qquad (1)$$

where $A_t$ is the activity at time t, U is the uptake rate, and S is the constant phosphate concentration (Harrison 1983). Thus a plot of ln $A_t$ versus time should be linear, with a slope equal to -U/S. Fig. 3 shows the least square lines fit to the log transformed data. The correlation coefficients were in all cases highly significant ($r^2 > 0.92$). Multiplying the slopes by the average phosphate concentrations yielded the uptake rates listed in Table 4.

Table 4. Phosphate uptake rates calculated from plots in Fig. 3 and average phosphate concentrations. Units are $\mu Md^{-1}$.

| Treatment | Uptake Rate |
|---|---|
| Control | 0.047 |
| Cycloheximide | 0.196 |
| Chloramphenicol | 0.016 |

## Discussion

Laws and Redalje (1982) reported the average photosynthetic rate of phytoplankton in the southeast sector of Kaneohe Bay to be 312 $\pm$ 46 mg $CM^{-2}d^{-1}$ during a one-year study following sewage diversion in May 1978. Since the average depth of the southeast sector is about 10 m, this figure corresponds to 31.2 $\pm$ 4.6 mg $Cm^{-3}d^{-1}$ or 2.6 $\mu mCd^{-1}$. If we assume a Redfield C:P ratio of 106 by atoms in the phytoplankton (Redfield et al. 1963), the implied P uptake rate is 0.025 $\mu Md^{-1}$, about half the value calculated for our control bottle. This calculation combined with the fact that addition of the prokaryote inhibitor chloramphenicol reduced the phosphate uptake rate by about a factor of three suggest that bacteria may indeed be responsible for a significant amount of the phosphate uptake in Kaneohe Bay. This conclusion must be qualified by the observations that (1) some phytoplankton (cyanobacteria) are prokaryotic (2) chloramphenicol can also affect eukaryotes through its effects on mitochondria and chloroplasts, and (3) there is naturally temporal variability in the photosynthetic rates of phytoplankton in Kaneohe Bay, and a factor of two discrepancy between the mean photosynthetic rate and the value measured on any given day is quite possible. Obviously further work would be needed to establish the role of bacteria in the P dynamics of Kaneohe Bay more quantitatively. The purpose of this experiment was primarily to introduce the students to methods and to provide some food for thought.

The effects of the cycloheximide might seem surprising if one anticipated that the principal effect would be to stop uptake by eukaryotic phytoplankton. However, since both uptake and regeneration seem to have been stimulated, the principal effect may have been to terminate zooplankton grazing. In the absence of such grazing, cyanobacteria and heterotrophic bacteria may have rapidly multiplied in numbers. Since phytoplankton do not regenerate nutrients, the increase in regeneration rate and higher phosphate concentration in the cycloheximide bottle would seem to indicate that heterotrophic bacteria also play an important role in phosphate regeneration.

To examine the effects of the cycloheximide treatment further, we partitioned the experiment into two time intervals, the first from the third to the twelfth hours, and the second from the twelfth to the twenty-fourth. Fig. 4 indicates that during the first time interval phosphate increased, and during the second time interval phosphate decreased. We calculated an uptake and regeneration rate for each time interval. The equations when the substrate concentration is not constant are (Harrison 1983)

$$r = \frac{\ln(AoSt/A_tSo)}{\ln(So/S_t).t}.(So - S_t) \qquad (2)$$

$$u = r + (S_o - S_t)/t \qquad (3)$$

where $A_t$ and $S_t$ are the activity and concentration, respectively, of phosphate at time t, and r and u are the regeneration and uptake rate. The results are given in Table 5. This analysis indicates that uptake rates were almost constant throughout the

Table 5. Phosphate uptake and regeneration rates for time intervals 3 - 12 h and 12 - 24 h in cycloheximide experiment with substrate concentration assumed to be a linear function of time during each time interval. Units are $\mu Md^{-1}$.

| Time Interval | u | r |
|---|---|---|
| 3 - 12 h | 0.166 | 0.368 |
| 12 - 24 h | 0.154 | 0.072 |

experiment, but that regeneration rates were much higher during the first time interval. What could be the cause of this behavior?

Without additional information, our interpretation of the results is speculative, but the following scenario seems consistent with the information at hand. The primary effect of the cycloheximide was to eliminate grazing by zooplankton and microzooplankton. As a result the bacterial populations increased. Bacteria, which have short generation times, responded rapidly. The bacteria consumed labile dissolved organics, in the process regenerating much phosphate. This process continued at a rapid rate for much of the first time interval, but slowed considerably during the second time interval, as the concentration of available organics declined. However, uptake continued at a high rate because of the elevated phosphate concentrations and because of the increased abundance of bacteria.

The implication of this scenario is that bacteria play an important role both as consumers and suppliers of phosphate. There is apparently much cycling of phosphate at the level of bacteria and phytoplankton. However, the zooplankton also play an important although somewhat indirect role, by consuming bacteria and phytoplankton and by supplying organic substrates which the bacteria subsequently utilize and in the process of utilization release phosphate. Additional work, undoubtedly including microscope work, would be needed to further examine these hypotheses.

Measurement of Phytoplankton Uptake and Excretion of Carbon (directed by Satoru Taguchi; data analysis by Margaret S. Murphy)

## Introduction

The $^{14}C$ technique for estimating primary production has been a common biological oceanographic tool for literally decades (Steemann-Nielsen 1951), but even today questions continue to be raised about exactly what the method measures (Peterson 1980; Dring and Jewson 1982), and whether standard incubation techniques create a serious bias in the results (Gieskes et al. 1979; Fitzwater et al. 1982). Although recent laboratory (Ditullio and Laws 1986) and field studies (Marra and Heinemann 1983) have helped to clarify some of the uncertainties about the method, several rather fundamental questions still remain.

The first of these questions concerns the meaning of the activity which appears in the particulate fraction after an incubation in the dark. The nature of this problem was dramatically illustrated in a paper by Hecky and Fee (1981) concerning primary production in Lake Tanganyika. They reported (p. 535) that, "Interpretation of the results...was confouneded by relatively high and variable rates of uptake in the dark, frequently higher than those at the highest illumination." By using autoradiographic methods, they discovered (p. 536) that, "a substantial portion of the uptake of $^{14}CO_2$ in the dark was done by organisms not active in the illuminated sample; most was associated with filmy detritus and large bacterial cells."

Methods for dealing with the problem of non-photosynthetic uptake of $^{14}C$ have included (a) ignoring the problem and simply calculating uptake based on light bottle incubations (Eppley and Sharp 1976), (b) subtracting dark bottle counts from light bottle counts (Davies and Williams 1984), (c) subtracting the counts obtained by filtering a sample immediately after adding the inorganic $^{14}C$ (Morris et al. 1971), and (d) subtracting the counts obtained from a bottle to which 10 μM DCMU has been added (Legendre et al. 1983). The rationale behind the last procedure is of some interest. Legendre et al. (1983) argue that uptake of $^{14}C$ in the dark does not, "provide proper values to be subtracted from light bottles, since dark $^{14}C$ fixation includes the effect of the Wood-Werkman reaction, which does not occur in the light bottles" (op. cit. p. 1003). They argued that addition of 10 μM DCMU to dark bottles might solve this problem, because, "in the dark, DCMU may act on ubiquinone (or coenzyme Q), an electron transport coenzyme analogous to plastoquinone. If DCMU does inhibit the activity of coenzyme Q, it would thus inhibit the Wood-Werkman reaction" (op. cit. p. 1002). On the other hand, addition of DCMU to the light bottle should block photosynthesis due to the effect of DCMU on photosystem II (Duysens and Sweers 1963), and in fact, "in a field experiment on natural populations, the $^{14}C$ incorporation with DCMU added was about 14% of the $^{14}C$ incorporation under saturating light" (Legendre et al. 1983, p. 1000). Thus if the Wood-Werkman reaction is solely responsible for the problem of correcting for non-photosynthetic $^{14}C$ uptake, addition of 10 μM DCMU to either light or dark

bottles could solve the problem.

A second perplexing problem concerns the extent to which phytoplankton excrete dissolved organic carbon (DOC). Williams (1981) has hypothesized that as much as 30% of the carbon fixed by phytoplankton is transfered directly via phytoplankton excretion to the DOC pool. Utilization of this DOC by bacteria, which in turn are grazed by protozoans and microzooplankton, has been viewed as an important "microbial loop" in marine food webs (Williams 1981; Azam et al. 1983). Recently this viewpoint has been challenged by Ducklow et al. (1986), whose studies indicated that most of the DOC taken up by heterotrophic bacteria was either respired or regenerated as DOC by organisms smaller than 1 μm. Ducklow et al. (1986) concluded, "Secondary (and, by implication , primary) production by organisms smaller than 1 micrometer may not be an important food source in marine food chains. Bacterioplankton can be a sink for carbon in planktonic food webs and may serve principally as agents of nutrient regeneration rather than as food."

It has occurred to many persons to use the $^{14}C$ technique to estimate rates of algal excretion of DOC. The idea is simple. One performs a standard $^{14}C$ incubation using inorganic $^{14}C$. One then looks for evidence of labeled DOC. The rate of production of DOC is calculated in the same way as particulate carbon (PC) production, but the activity in the DOC is substituted for the activity in the PC. Using this technique, most experimentalists have obtained DOC excretion rates of about 5 - 10% of the

corresponding photosynthetic rate (e.g. Smith et al. 1977). One obvious problem with this approach is that DOC excreted by phytoplankton and subsequently assimilated by bacteria would still appear in the PC fraction. Hence the activity in the DOC represents only those substances which were excreted but not subsequently assimilated by bacteria, and could thus grossly underestimate actual excretion rates. Cole et al. (1982) used a serial filtration technique to try to separate algal and bacterial particulate $^{14}C$ activity, and concluded, "The amount of PDOC (photosynthetically produced DOC) measured in the water is smaller than the amount actually released by phytoplankton because microbes metabolize PDOC rapidly. Failure to account for microbial utilization could lead to a serious underestimate of gross PDOC during measurements of primary production. Burney (1986) studied diel cycles in dissolved carbohydrate concentrations, and observed a steady increase during the day and decline at night. He attributed the decline to "rapid bacterial metabolism," and concluded that, "The source of this carbohydrate appears to be the extracellular release of phytoplankton photosynthate fixed during the recent photoperiod. Observed rates require substantially greater primary production than indicated by the $^{14}C$ method."

On the other side of the coin, Sharp (1977) has argued that, "Evidence of extensive excretion by phytoplankton is not good." In field experiments, he argues that there has been inadequate assessment of control blanks. The problem is that in order to measure to DOC activity, one must separate the DOC from the

inorganic carbon (IOC). Since both are dissolved in the water, filtration will obviously not do the job. The standard procedure has been to acidify and bubble an aliquot of sample filtrate to remove the IOC. However, since the DOC activity is typically less than 0.01% of the IOC activity, this stripping procedure must be extremely efficient if the DOC counts are not to be obscured by residual IOC activity. Furthermore, if the IOC contains only a tiny fraction of labeled DOC contaminants, the activity of the photosynthetically produced DOC could be obscured by the contaminants. Obviously appropriate measures are needed to deal with these possible problems if algal excretion rates are to be properly assessed.

## Material and Methods

A surface water sample was collected with a plastic bucket at approximately sunrise on July 2, 1985, at station 1 in the southeast sector of Kaneohe Bay (Fig. 1). The water was screened through 183 μm nytex screening to remove large zooplankton. The $^{14}C$ - $NaHCO_3$ stock solution, with an activity of 100 μCi/ml, was prefiltered through a 0.2 μm Nuclepore filter to remove any particulate $^{14}C$ activity. The seawater was dispensed into light and dark 4.4 l polycarbonate bottles, and inoculated with a total of 400 μCi of $^{14}C$ - $NaHCO_3$. The bottles were incubated under natural light conditions for a total of 24 h in a water table with flowing surface seawater to provide temperature control. At time zero (7 AM) one ml aliquots were taken from each bottle and mixed with one ml of $CO_2$ trapper (phenethylamine) in a

scintillation vial. These one ml samples were intended to provide a measure of the activity added to the bottles.

The bottles were sampled at time zero and at 6, 12, and 24 h. Duplicate 250 ml aliquots were filtered through 10 μm Nuclepore filters to remove the largest phytoplankton. Duplicate 200 ml aliquots of the 10 μm filtrate were then filtered through 2 μm Nuclepore filters to isolate the nanoplankton smaller than 10 μm (Sieburth et al. 1978). Finally duplicate 100 ml aliquots of the 2 μm filtrate were filtered through 0.2 μm Nuclepore filters to isolate the picoplankton (Sieburth et al. 1978). In the same manner, duplicate 250 ml, 200 ml, and 100 ml samples were collected on filters to determine the concentration of cholorphyll *a* (chl *a*) in the >10 μm, 2 - 10 μm, and 0.2 - 2 μm size fractions, respectively. The filters for $^{14}C$ analysis were placed in labeled plastic scintillation vials, and one ml of 1.2N HCL added to drive off any residual inorganic $^{14}C$. Ten ml of ACS scintillation cocktail (Amersham) were then added, and the samples stored in the dark for 24 h before counting on a Packard Tri-Carb ® model 4640 liquid scintillation counter. Production rates were calculated using the equations in Strickland and Parsons (1972) and an assumed IOC concentration of 26 mg/l. The filters for chl *a* analysis were placed in labeled film containers containing 5 ml of 100% acetone and stored at -20°C prior to analysis. Chl *a* concentrations were measured fluorometrically with phaeopigment corrections made following the recommedation of Holm-Hansen and Riemann (1978) for the acidification step. The activity of organic carbon in the light bottle filtrates was

assessed both for the 2 m and 0.2 m filtrates. This activity included both DOC activity and (certainly in the case of the 2 m filtrate) PC activity. Ten ml aliquots of filtrate were placed in plastic vials and acidified to pH 2.8 with 1N HCl. The vials were then bubbled with air for 40 minutes. One, two, and four ml aliquots were then taken from the 10 ml acidified sample and placed in liquid scientillation vials for counting.

At the 24 h time point, a total of 750 ml of filtrate from the 2 m and 0.2 m light bottle filters were collected and mixed in five different proportions as indicated in Table 6. Fifty ml aliquots of each treatment (i.e. mixing ratio) were dispensed into three light and three dark bottles. One light and dark bottle pair received chloramphenicol, a metabolic inhibitor which acts only on bacteria, mitrochondria, and chloroplasts by inhibiting peptidyl transferase of large sub-units (Dixon and Webb 1979). The third light and dark bottle pair served as controls. After a twelve-hour incubation (roughly sunrise to sunset) duplicate 10 ml aliquots were filtered on 0.2 m Nuclepore filters and assayed for particulate $^{14}C$ activity as previously described.

## Results

The one ml aliquots taken for total activities at time zero yielded values of 1.49 and 1.15 x $10^5$ dpm per ml in the light and dark bottles, respectively. The expected activity in each bottle was $(4)(2.22 \times 10^8)/[(4.4)(1000)] = 2.02 \times 10^5$ dpm. A subsequent check of the activities in the $^{14}C$ ampules revealed that they did

Table 6. Design of experiment utilizing various proportions of 24h 2 µm and 0.2 µm filtrate. Total volume of each treatment was 300 ml.

| Treatment | % 2 µm Filtrate | % 0.2 µm filtrate |
|---|---|---|
| 1 | 0 | 100 |
| 2 | 25 | 75 |
| 3 | 50 | 50 |
| 4 | 75 | 25 |
| 5 | 100 | 0 |

contain the expected activities. Therefore both because the one ml counts in the light and dark bottles differed by 30% and because even the higher of the two counts is 25% lower than the expected activity, we chose to disregard these one ml counts and assumed that both bottles did in fact contain $2.02 \times 10^5$ dpm/ml. The most likely cause of the low and poorly replicated one ml counts is lack of a homegenous solution in the scintillation cocktail (P. leB. Williams, pers. comm.).

The activities retained on the filters are listed in Table 7. The median coefficient of variation (i.e. 100 x standard deviation/mean) of the duplicate counts was 16%. Using the equations in Strickland and Parsons (1972) and an assumed total activity at time zero of $2.02 \times 10^5$ dpm/ml, we calculate that each of the numbers in Table 7 should be multiplied by 0.1351 to convert the values to mg $C/m^3$. These values are listed in Table 8. We subtracted dark bottle counts from light bottle counts in order to obtain the production numbers. Integral averages over the photoperiod of the chl *a* concentrations in each size fraction are listed in Table 8. Productivity indices (PI's) during the photoperiod (0 - 12 h) were calculated by dividing the photoperiod production values by the corresponding chl *a* values. These ratios are also listed in Table 8.

The activities in the 0.2 μm and 2 μm filtrates are listed in Table 9. Three of the values were unrealistically high and were excluded from the subsequent data analysis. A linear regression of dpm versus volume of aliquot yielded a slope with

Table. 7. Average of activities of $^{14}C$ in each ml of filtered sample during 24 h incubation. Values are means of duplicate light (L) and dark (D) bottle counts. Results are reported as disintegrations per minute (dpm) per ml.

| Time | Size Fraction | | | | | |
|---|---|---|---|---|---|---|
| | 0.2 - 2 μm | | 2 - 10 μm | | > 10 μm | |
| (Hours) | L | D | L | D | L | D |
| 0 | 1.44 | 1.48 | 0.12 | 0.16 | 0.09 | 0.11 |
| 6 | 150.19 | 27.40 | 84.37 | 8.75 | 35.39 | 4.53 |
| 12 | 180.43 | 24.45 | 87.90 | 9.17 | 62.62 | 7.87 |
| 24 | 204.62 | 42.90 | 88.91 | 17.78 | 75.11 | 7.79 |

Table 9. Activities in filtrates from 0.2 μm and 2 μm filters. Results reported as dpm in excess of blank counts, which were 55.8 dpm.

| Time (h) | Filter Size (μm) | Volume of Aliquot (ml) | | | dpm/ml from regression | correlation coefficient |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 4 | | |
| 0 | 0.2 | 253* | 11 | 73 | | |
| | 2.0 | 0 | 18 | 55 | 21.8 | 0.993 |
| 6 | 0.2 | 12 | 86 | 166 | 50 | 0.986 |
| | 2.0 | 74 | 182 | 426 | 118 | 0.9996 |
| 24 | 0.2 | 0 | 1179* | 2088* | | |
| | 2.0 | 0 | 22 | 105 | 36 | 0.991 |

*Excluded from analysis.

units of dpm/ml, and those values are reported in Table 9. At time zero there should theoretically have been no difference in the activities in the 0.2 and 2 μ m filtrates, and the values for each aliquot volume were therefore averaged at that time point in performing the linear regression.

The activities at the end of the photoperiod on 7/3/85 in the control and metabolic inhibitor experiments are shown in Table 10. In order to intelligently interpret these data, it is necessary to know the activities in the various fractions at the beginning of the photoperiod. From Table 7 it is apparent that the activity in the 2 μm filtrate must have been 205 dpm/ml in the light bottle. Obviously the activity in the 0.2 μm filtrate which was retained on a 0.2 μm filter at sunrise on 7/3/85 was by definition zero. Making these time zero adjustments, we calculated the production values listed in Table 11. The chl*a* concentration in the light bottle 2 μm filtrate at sunrise on 7/3/85 was 0.37 $mg/m^3$.

## Discussion

At first glance the results of the simple $^{14}C$ uptake experiment seem straightforward and reasonable. The picoplankton (0.2 - 2 μm) accounted for about half the total uptake and 40% of the chl*a* and organisms passing a 10 μ m filter accounted for about 80% of total uptake and 90% of the chl *a*. The latter result is similar to results reported by McCarthy et al. (1974) in another estuary (Chesapeake Bay), where the <10 μm fraction accounted for 81% of the chl*a* and 95% of the productivity. The productivity index

(PI) for the entire phytoplankton community was 9.7g $Cg^{-1}chlh^{-1}$ during the photoperiod, a result very consistent with the results of other studies in Kaneohe Bay (Laws and Redalje 1982; Laws et al. 1984).

However, a closer examination of the data reveals at least one disturbing problem. In the picoplankton and <10 μm size fractions, the calculated rate of carbon assimilation was positive at night, and in the case of the <10 μm fraction amounted to 23% of the photoperiod uptake. Clearly this nighttime assimilation cannot represent photosynthesis. Since dark bottle counts were subtracted from light bottle counts, this behavior presumably does not reflect processes such as the Wood-Workman reaction. One possible explanation is that labeled DOC compounds were excreted by the phytoplankton during the day, and subsequently assimilated by phytoplankton and/or bacteria at night. Thus the apparent incorporation of $^{14}C$ into particulate material during the night would in fact represent uptake of carbon that was converted to organic form during the day but remained as DOC rather than PC until sometime during the night.

Another puzzling aspect of the results is the fact that the PI ratio for the 0.2 - 10 μm size fraction (5.3) was less than half the PI ratio in the picoplankton (13.2) and <10 μm fraction (16.4). This result may be real, but intuitively one would be inclined to expect a monotonic trend (or no trend) in PI ratios as a function or organism size, as is the case with overall metabolic rates (Banse 1976).

Finally it seems curious that photosynthetic rates during the afternoon were so much lower than during the morning in the picoplankton and 2 - 10 m fractions. Both Malone (1971) and Paerl and Mackenzie (1977) have reported diel periodicity in carbon fixation patterns among nannoplankton and netplankton, and it is true that under some conditions the nannoplankton tend to exhibit a morning peak in photosynthetic rate, while the netplankton either show an afternoon peak or at least contribute more to overall $^{14}C$ uptake in the afternoon than in the morning. While such observations are qualitatively consistent with our results, the decline in productivity in the picoplankton and 2 - 10 m fractions is so great as to suggest bottle confinement problems. An alternative explanation is that for some reason the 6h activities were systematically high, an error which would have caused the morning production to be overestimated and the afternoon production to be underestimated by the same amount.

Phytoplankton excretory products would of course be found in the 0.2 m filtrate, and from Table 9 it is clear that the only time interval over which excretion rates could be estimated was the first six hours of the photoperiod. The increase in DOC activity during that period was 50 - 21.8 = 28.2 dpm/ml, which corresponds to a production of 3.8 $mgC/m^3$. The total photosynthetic production over the same time interval was 31 $mgC/m^3$. Hence excretion appears to have been (100)(3.8)/(3.8 + 31) = 11% of the corresponding photsynthetic rate. This figure is quite consistent in magnitude with many literature valves (e.g. Smith et al. 1977). However, a close examination of Table 9 reveals a

disturbing problem. The difference between the 0.2 and 2.0 μm activities in Table 9 should equal the activity in the picoplankton size fraction in Table 7. For example, at six hours this difference should be 150 dpm/ml, and at 24h the difference should be 205 dpm. In fact however, the difference at 6h is only 118 - 50 = 68 dpm/ml, and at 24h the difference is less than 36 dpm/ml. What is the cause of this discrepancy?

Certainly one problem may simply have been statistical. In Table 7 the activities in the picoplankton fraction were estimated by filtering 100 ml of water. In Table 9 the activity in at most four ml was counted. Thus the statistics are much better for the Table 7 data. One is obviously led to wonder how reliable the 11% excretion rate figure is. This problem of measuring low activities in only a few ml of sample is one of the problems which has cast doubt on some of the reported excretion rates, a point raised by Sharp (1977). One solution is to first dirve off the inorganic $^{14}C$ from a large volume of filtered water, and then oxidize the DOC by wet combustion (Fogg et al. 1965). The oxidized $^{14}C$, now inorganic, is then driven off by acidification and concentrated in a suitable trap. Obviously this procedure is more tedious than counting small aliquots directly, and also requires a knowledge of the oxidation and trapping procedure efficiency.

The results of the metabolic inhibitor studies produced some informative and in some ways puzzling results. $^{14}C$ uptake essentially came to a halt with the addition of cycloheximide

(Table 11), with light bottle and dark bottle counts being little different. This result is quite informative when compared to the phosphate uptake experiment, in which cycloheximide addition appeared to stimulate both uptake and regeneration. The implication is that bacteria, which are presumably not affected by cycloheximide, are important in both uptake and regeneration of phosphate. Judging from the effects of cycloheximide on $^{14}C$ uptake, the stimulated uptake of phosphate which resulted from the addition of cycloheximide could not have resulted from phytoplankton uptake.

The chloramphenicol results are equivocal. At four of the five dilution ratios the chlorampenicol light bottle uptake was either equal to or less than the control uptake (Table 11), but the results show no consistent pattern with mixing ratio. One might have expected the chloramphenicol to reduce $^{14}C$ uptake to the extent that cyanobacteria contribute to photosynthesis. However, the data are too scattered to reach any firm conclusions.

The control results themselves were surprising. We had expected to see a linear relationship between the percentage of 2 μm filtrate and $^{14}C$ uptake, but at the first three mixing ratios $^{14}C$ uptake was essentially constant. Furthermore, there was apparently a significant production of $^{14}C$-containing particles which could be retained on a 0.2 μm filter even in the 0.2 μm filtrate!

On the other hand, if we simply draw a regression line through

the control data (Fig. 5), the line passes almost exactly through the origin, and the slope implies a production of 163 $mgC/m^3$ for the picoplankton. Since the picoplankton biomass at sunrise on 7/3/85 was 0.37 $mg/m^3$, the implied PI is (163)/[(12) (1.37)] = 36.7 g C $g^{-1}$ chl *a* $h^{-1}$. Although the theoretical maximum PI is only about 25 (Falkowski 1981), the figure of 36.7 is not unreasonable if the picoplankton increased by over a factor of two during the first six hours of the incubation on 7/2/85. We conclude that in fact the 0.2 μm filtrate contributed nothing to $^{14}C$ uptake, and that the picoplankton assimilated about 163 $mgC/m^3$ in the control incubation on 7/3/85.

Table 10. Average activities found on duplicate 0.2 µm filters after photoperiod incubation with indicated proportions of 0.2 µm and 2 µm filtrate beginning at approximately sunrise on 7/3/85. Results are reported as dpm/ml.

Control

| % 0.2 µm filtrate | % 2 µm filtrate | Light Bottle | Dark Bottle |
|---|---|---|---|
| 100 | 0 | 311 | 82 |
| 75 | 25 | 325 | 92 |
| 50 | 50 | 450 | 129 |
| 25 | 75 | 677 | 143 |
| 0 | 100 | 1830 | 251 |

Chloramphenacol

| | | | |
|---|---|---|---|
| 100 | 0 | 309 | 35 |
| 75 | 25 | 47 | 56 |
| 50 | 50 | 1138 | 85 |
| 25 | 75 | 170 | 103 |
| 0 | 100 | 1424 | 87 |

Cycloheximide

| | | | |
|---|---|---|---|
| 100 | 0 | --- | 65 |
| 75 | 25 | 143 | 105 |
| 50 | 50 | 179 | 141 |
| 25 | 75 | 203 | 234 |
| 0 | 100 | 257 | 292 |

Table 11. Production values for photoperiod incubations of 7/3/85. Values are $mgC/m^3$.

| % 0.2 µm filtrate | % 2 µm filtrate | Light Bottle | Dark Bottle |
|---|---|---|---|
| | | Control | |
| 100 | 0 | 42 | 11 |
| 75 | 25 | 37 | 6 |
| 50 | 50 | 47 | 4 |
| 25 | 75 | 71 | -1 |
| 0 | 100 | 220 | 6 |
| | | Chloramphenicol | |
| 100 | 0 | 42 | 5 |
| 75 | 25 | 0 | 1 |
| 50 | 50 | 140 | -2 |
| 25 | 75 | 2 | -7 |
| 0 | 100 | 165 | -16 |
| | | Cycloheximide | |
| 100 | 0 | --- | 9 |
| 75 | 25 | 12 | 7 |
| 50 | 50 | 10 | 5 |
| 25 | 75 | 7 | 11 |
| 0 | 100 | 7 | 12 |

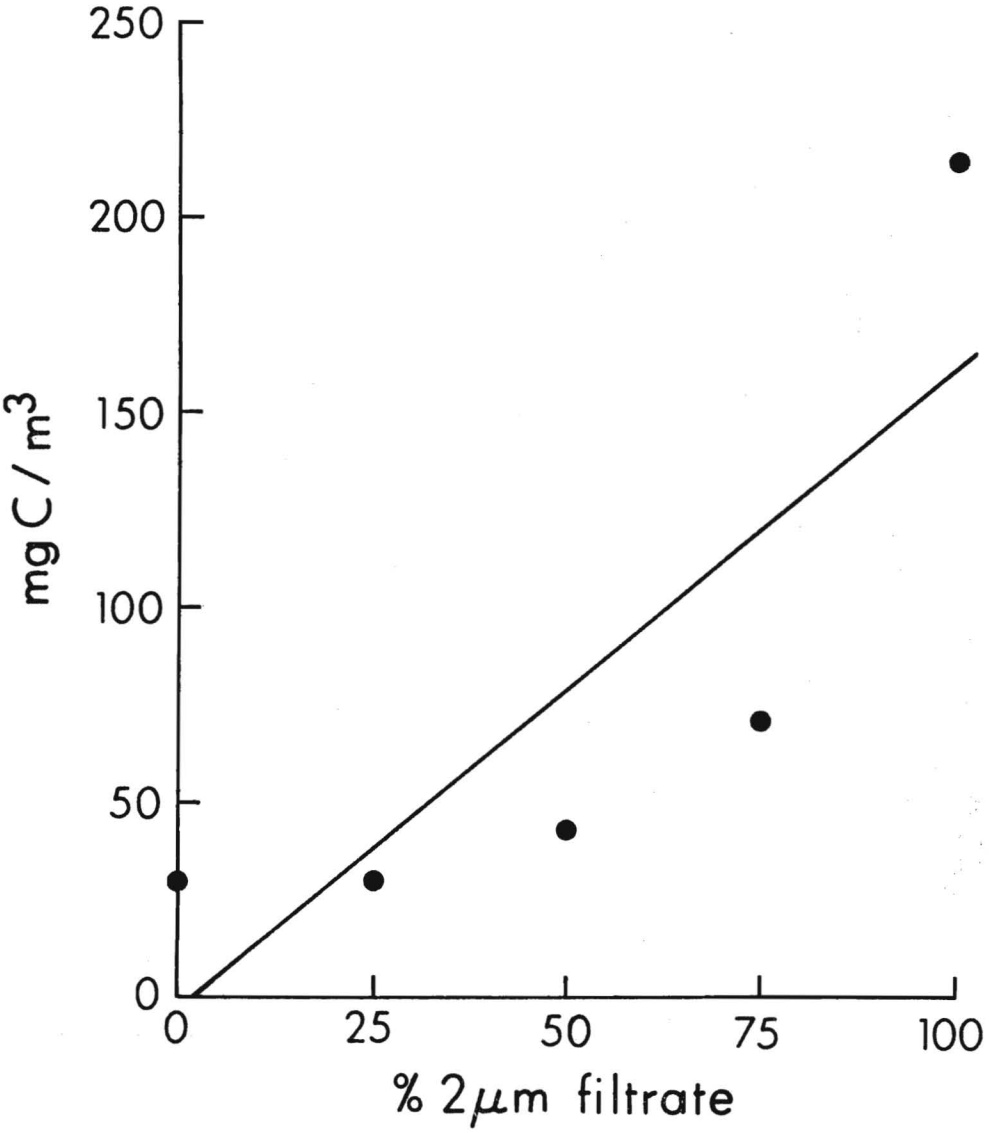
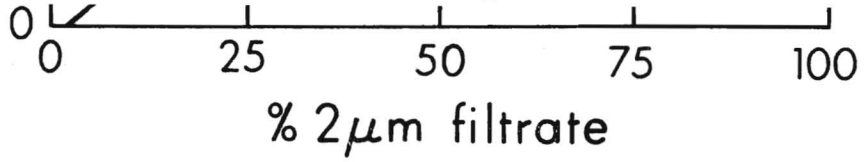



Fig. 5. Photosynthetic production after 12h incubation in control flasks containing mixtures of 0.2 μm and 2 μm filtrate.



Using Epifluorescent Microscopy and Fluorescent Microspheres to Measure the Grazing Rate of the Ciliate Cyclidium on Bacteria (Megan D. Bailiff, with advice from Mike Pace).

## Introduction

Over the last ten years the traditional paradigm concerning energy flow through the pelagic food web has begun to change. Pomeroy (1974) stated a decade ago that "there is increasing evidence that zooplankton are not metabolically dominant. Microorganisms (whose biomass is approximately equal to that of net plankton) are greater movers of energy and materials because of their higher metabolic rate per unit mass". This idea of microorganisms' being major contributors to the complex food web of the ocean has become an important issue in relation to the pathway of energy and material flow in plankton (Pomeroy 1974; Sieburth 1976; Williams 1981; Azam et. al. 1983; Ducklow 1983; Landry et. al. 1984). The standing stocks and metabolism suggest high rates of growth, yet there appears to be only moderate changes in standing stock (Ducklow and Kirchman 1983; Laws et. al. 1984). Hence, it is speculated based on recent research that planktonic protozoans exert a heavy grazing pressure on picoplankton populations.

Laboratory-determined feeding rates and measures of protozoan abundance, particularly heterotrophic flagellates, seem to show that protozoa may actually be capable of controlling picoplankton populations (Fenchel 1982 b & c). Unfortunately little is known

about the behavior of these organisms in situ, and reliable in situ grazing rate measurements are not currently available. Thus, it is not yet possible to support or reject the hypothesis of protozoan grazing pressure on picoplankton.

However, interest in a new hypothesis often stimulates the creation of new methods for measuring relevant parameters. The question of how to measure in situ the quantity and type of protozoans which are grazing on picoplankton has resulted in the development of several new methods for measuring grazing rates under oligotrophic conditions. These methods include the use of radiotracers, inhibitors, dilution, filtration and fluorescent microspheres. The final method, namely using epifluorescent microscopy and fluorescent microspheres to measure protozoan grazing on bacteria, was the method dealt with in this study. This method offers the potential of making in situ measurements with a minimum of sample manipulation and time. It is necessary, however, to begin by examining the method carefully and critically to determine if the underlying assumptions are valid and supportable.

Several assumptions are made when applying the fluorescent microsphere technique. First, it is assumed that the distribution of the microspheres is similar to that of the picoplankton throughout the experiment. Namely, it is assumed that there is no clumping of the microspheres, nor significant settling or differential absorption of the particles. Second, it is assumed that there is no egestion of the microspheres during the incubation time of the experiment. The egestion time must be

determined or known prior to performing an experiment in order to choose a suitable incubation time. Third, it is assumed that all protozoans which feed on picoplankton will ingest the microspheres. The final assumption is that microspheres are ingested in proportion to their density, and that there is no discrimination between microspheres and picoplankton. Several experiments were performed to test the validity of these assumptions and to assess the relative accuracy, precision and sensitivity of the microsphere method in measuring protozoan grazing rates on bacteria.

## Materials and Methods

Experiments were performed on samples taken from a two-stage continuous culture system which was set up to run at near oliogotraphic ocean conditions. The continuous culture system consisted of a medium-fed first-stage chamber where bacteria were cultured and two second-stage chambers which received overflow from the first. The medium consisted of sterile-filtered (0.22 μm) seawater enriched with f/2 nutrients (Guillard and Ryther 1962) supplemented with casamino acids (Sigma Chemical) at 2 $mgl^{-1}$ and surcrose at 1.1 $mgl^{-1}$ as carbon sources. One of the second-stage chambers acted as the control while the other was inoculated with the ciliate Cyclidium and served as the grazing (experimental) chamber. The continuous culture apparatus and the medium were autoclaved and set up in a dark refrigerator and kept at 19.5°C. After medium had been allowed to flow completely through the system, the grazing chamber was inoculated with 1 ml

of ciliate culture. Once the system acheived steady state conditions, a grazing experiment could be run.

The whole system was monitored daily, a process which entailed measuring the bacterial concentrations in all three chambers, the ciliate concentration in the grazing chamber, and the turnover volume of the two second-stage chambers. Daily samples of 5 ml were taken from the three chambers and were preserved immediately with 0.5 ml of 20% formalin. These samples were prepared for enumeration within an hour of their collection using the fluorescent dye acridine orange (A.O.). One ml of a filtered seawater (fsw)/formalin solution and 1 ml of sample stained for two minutes with 0.2 ml of A.O. were drawn through a 0.22 µm Nuclepore filter [previously stained with Irgalan black solution to help eliminate background fluorescence (Hobbie et. al. 1977)] at a vacuum no greater than 200 torr. The Nuclepore filter was backed with a GF/C glass fiber filter to promote even dispersion of the sample. Both these filters had been soaked for several minutes in distilled water to remove the excess stain from the Nuclepore filter and to allow more even suction on the glass fiber filter. After filtration, the filter was rinsed once with 1 ml of the fsw/formalin solution. The filter was then removed, allowed to dry, and placed on a drop of immersion oil (Cargille type A) on a glass slide, specimen side up. One drop of oil was placed on the center of the filter, followed by a cover slip. Samples were usually counted immediately after preparation. If immediate counting was not possible the slides were frozen and counted within 24 hours after preparation.

The procedure for the grazing experiment (Fig. 6) was as follows: 80 ml was drawn from the grazing chamber of the continuous culture system. This subsample was inoculated with a tracer amount of microsphere standard of known density to achieve a final bacteria:microsphere ratio of 5:1. The total incubation time of the experiment was thirty minutes with triplicate subsamples (5 ml) being taken at time zero, fifteen and thirty minutes to determine the number of microspheres present in ciliate guts. Triplicate subsamples (5 ml) were also taken at the end of the incubation time and used to determine the actual bacteria:microsphere ratio of the final experimental solution. The ratio subsamples were processed and mounted using the same technique as described above (0.4 ml A.O. was used to stain 5 ml of sample). The time course subsamples were stained using DAPI, a highly specific and sensitive fluorescing DNA stain. DAPI was used in order to facilitate searching under the FT 395 filter, which illuminates only the blue stained ciliates, and then switching to the FT 519 filter, under which the microspheres could be seen and counted. This procedure allowed for a relatively unbiased enumeration of the ingestion rate of the ciliates on the microspheres. The time course subsamples (5 ml) were stained with 0.4 ml DAPI for 5 minutes. They were then drawn through 1 - 2 µm Nuclepore filters (again backed with GF/C filters) in order to retain only the ciliates as much as possible. The filters were then rinsed twice with 1 ml aliquots of the FSW/formalin solution to remove any unconsumed particles still on the filter.

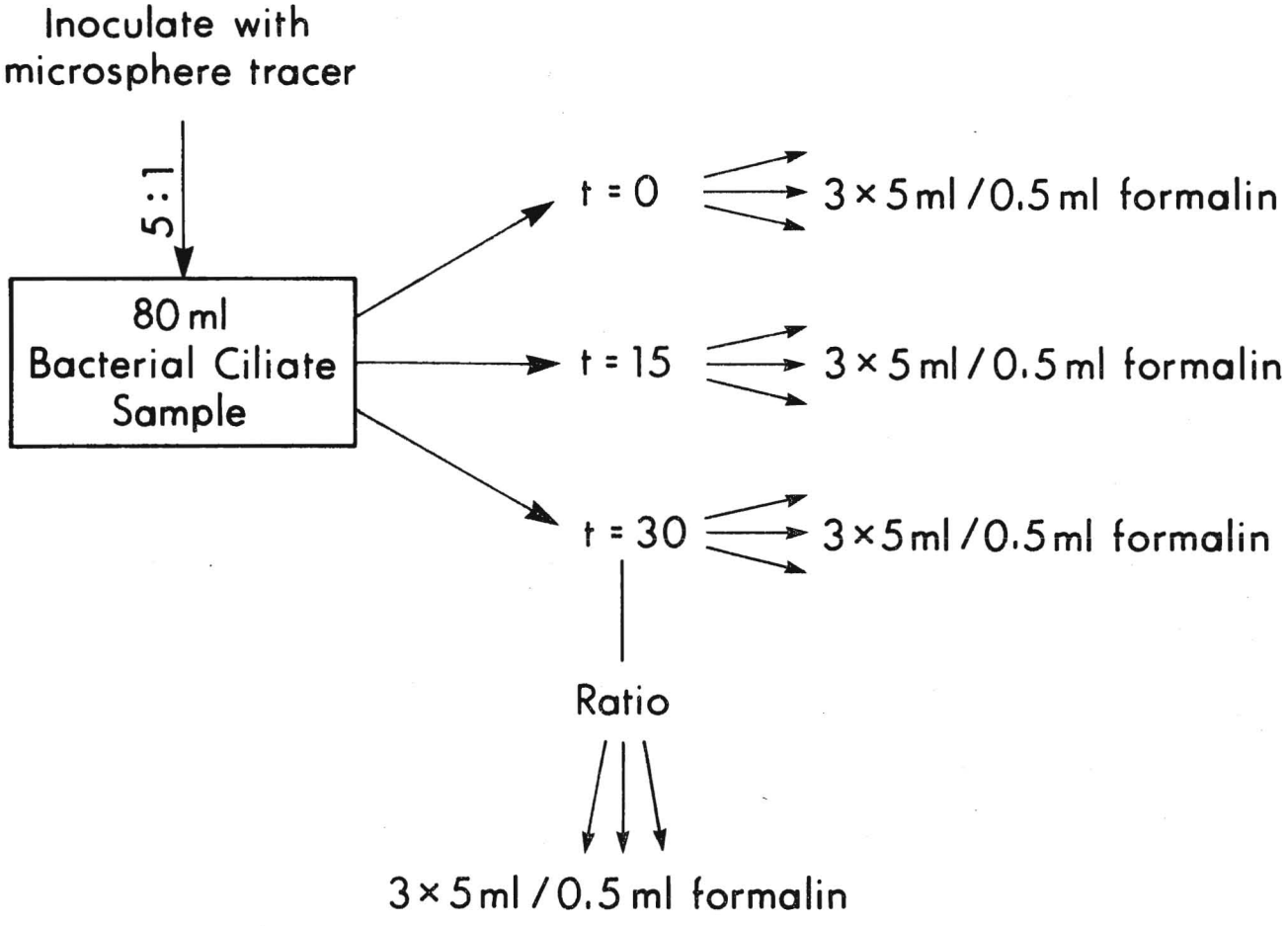



Fig. 6. Diagram of procedure followed for protozoan grazing experiments.

A Zeiss standard microscope was used with a 100-W HBO mercury light source. For the A.O. counts a G436 exciter filter, FT510 dichromatic beam splitter, LP520 barrier filter and a Planachromat 100/1.25 oil with iris objective were used. For counts with DAPI a G365 exciter filter, FT395 chromatic beam splitter, LP420 barrier filter and Neofluar 100/1.30 oil objective were used. The Neofluar objective without flat field correction must be used for DAPI fluorescence because the lens coatings of the optically superior Planachromat absorb in the excitation range of the DAPI filter set. For all of the bacteria counts 10 grids (10 x 10 μm) per filter were enumerated. In the case of low bacteria counts in the ciliated chamber, 20 grids were counted, so that at least 100 individual cells were counted per filter. For the ciliates, 10 fields were counted per filter using the Planachromate 10/0.22 objective.

## Results

The latex microspheres used in the grazing experiments were a uniform 0.6 μ m in size. To test the first assumption standards of the microspheres were prepared in both FSW and distilled water and the amount of clumping was observed over a two hour period. In a high concentration standard the microspheres do tend to clumb in FSW rather rapidly. The clumping was considerably less in the distilled water standards even after several hours. To curtail the clumping even further the microspheres were treated with the protein BSA, and the stocks, made up in distilled water, were stored in the refrigerator. It is best to make up the

microsphere standards in FSW just prior to an experiment to decrease the chances of clumping as much as possible. At experimental levels, $10^4$ to $10^6$ spheres per ml, the microspheres tend to remain well dispersed for several hours.

The incubation time for the grazing experiments was usually 15 minutes. It has been shown that the cell passage time for the microspheres is less than thirty minutes, but that linear ingestion rates are obtained over a fifteen minute incubation (Pace, per. comm.). At low bacterial concentrations (i.e. oligotrophic ocean conditions) the cell passage time of microspheres may be longer. Obviously, incubations must be kept sufficiently short to avoid egestion. It was assumed that under artificial oligotrophic conditions 15 minutes was a satisfactory incubation time. In one case data were collected at both 15 and 30 minutes to check for any evidence of nonlinearity in the results.

The most important question being asked regarding the microsphere method is whether all the protozoans will ingest the microspheres and bacteria at similar rates. It is documented that ciliates have little or no capability to discriminate between small particles by properties other than size (Fenchel 1980 a,b). To test the hypothesis of non-selectivity (assumptions 3 - 4) the ratio of microspheres to bacteria was varied in separate grazing experiments. The null hypothesis is that ingestion rates will be the same at different bacteria to microsphere ratios. Alternatively, if there is selectivity against the microspheres then the ingestion rates may be lower in suspensions containing a

Table 12. Ciliate grazing rates calculated at two bacteria/microsphere ratios.

| Bacteria/microsphere ratio | | Microspheres per ciliate | | Grazing Rate (Bacteria/Ciliate-h) | |
|---|---|---|---|---|---|
| Nominal | Experimental | 0 | 15 min | | mean SD |
| 10 | 11.0 | 0.36 | 1.88 | 66.9 | |
| | 11.9 | 0.4 | 1.02 | 27.1 | 43.5 ± 20.8 |
| | 10.0 | 0.24 | 1.15 | 36.4 | |
| 5 | 4.0 | 0.16 | 2.55 | 38.2 | |
| | 5.5 | 0.26 | 2.43 | 47.7 | 39.7 ± 7.4 |
| | 5.5 | 0.28 | 1.79 | 33.2 | |

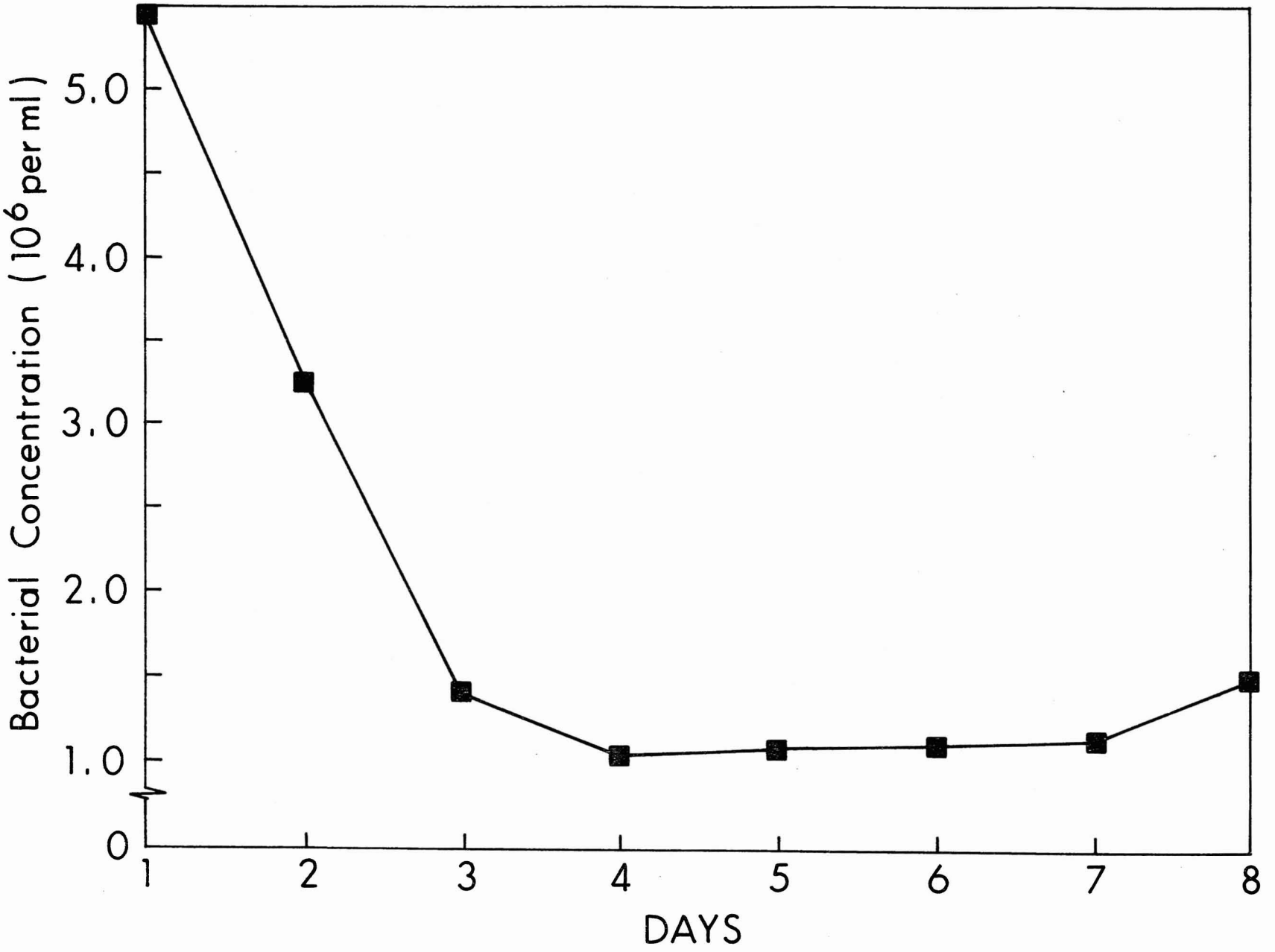



Fig. 7. Bacterial concentrations in the ciliate chamber versus time

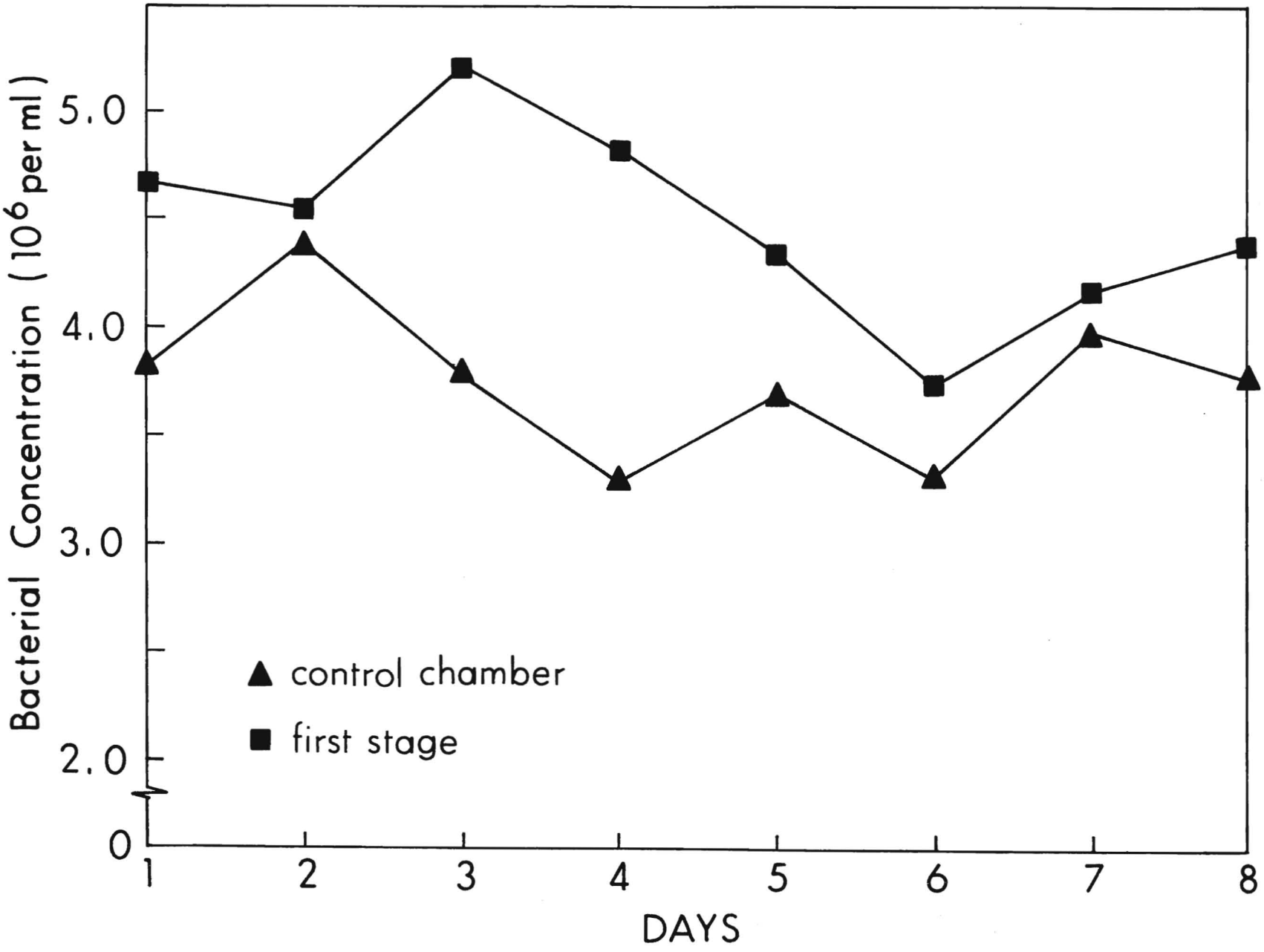



Fig. 8. Bacterial concentrations in the control and first

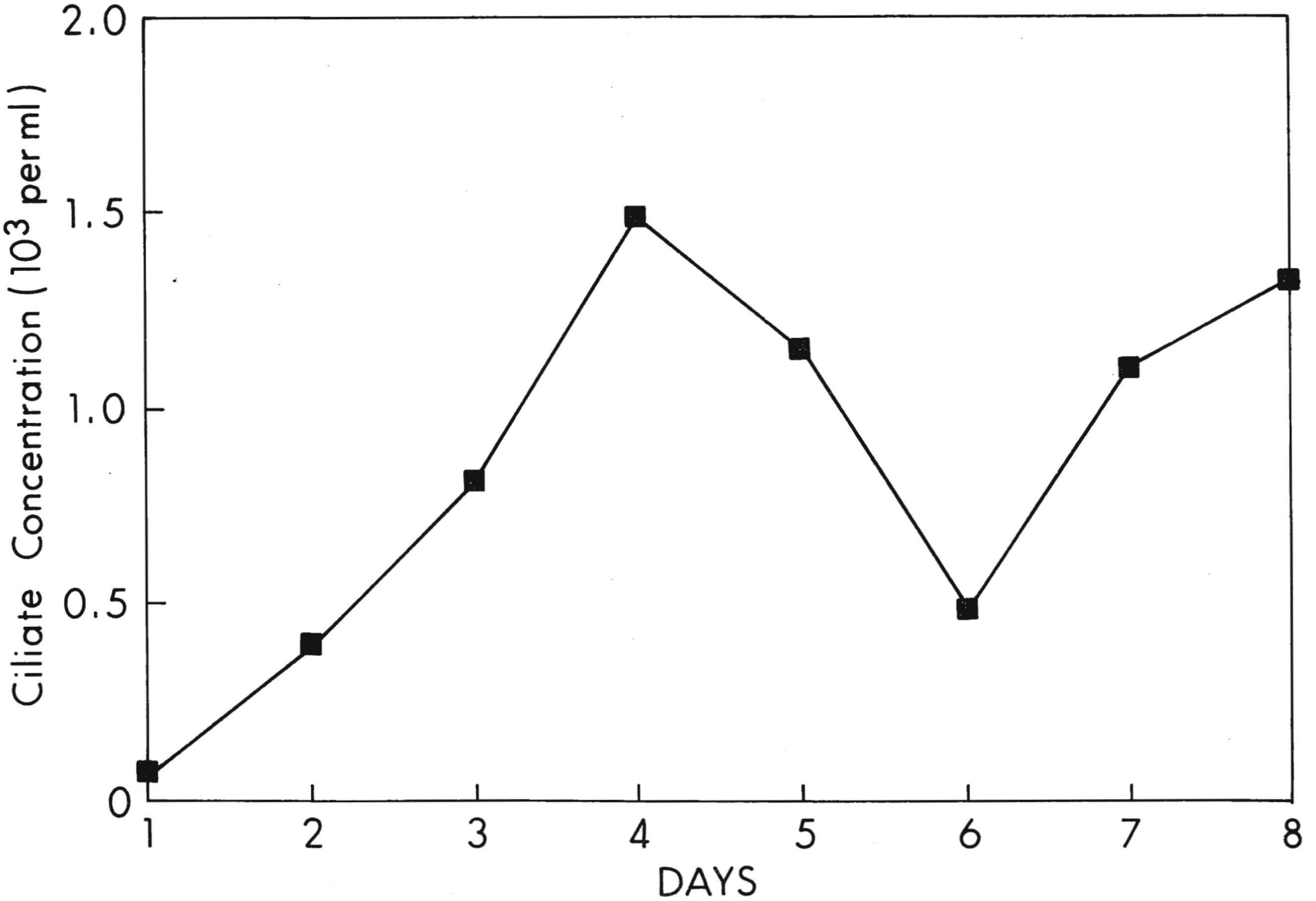



Fig. 9. Ciliate concentrations in the ciliate chamber versus

Table 13. Grazing rates calculated from continuous culture studies (days 5 - 10) and microsphere experiment (day 10). The bacteria/microsphere ratio was 5 in the microsphere experiments. Grazing rates are bacteria per ciliate per hour.

Microsphere Experiment

| Microspheres per ciliate | | | Grazing Rate | |
|---|---|---|---|---|
| 0 | 15 min | 30 min | 0 - 15 min | 0 - 30 min |
| 0.12 | 3.39 | 6.51 | 65.4 | 63.9 |
| 0.10 | 3.52 | 7.17 | 68.4 | 70.7 |
| 0.14 | 3.21 | 6.95 | 61.4 | 68.1 |
| | mean ± SD | | 65.1 ± 3.5 | 67.6 ± 3.4 |

Continuous Culture Results

| Day | Grazing Rate |
|---|---|
| 4 | 56.6 |
| 5 | 87.3 |
| 7 | 98.9 |
| 8 | 65.6 |
| 9 | 72.2 |
| 10 | 46.6 |
| mean ± SD | 71.2 ± 19.4 |

higher ratio of microspheres to bacteria. Two experiments were performed to test this assumption, the first was run at a ratio of bacteria:microspheres of 5:1 and the second at a ratio of 10:1. There was no significant difference (t-test, $p > 0.2$) between the grazing rates of the ciliates at the different ratios (Table 12), but the standard deviations of the experimental results would have precluded seeing a statistically significant (i.e. $p < 0.05$) difference unless the two mean grazing rates had differed by more than 35 bacteria per ciliate-h.

The results of the continuous culture studies are shown in Figs. 7 - 9 and Table 13. The bacterial counts in the ciliate chamber were reduced to a low and constant level of about $10^6$/ml by day four (Fig. 7) and the system was assumed to be in steady state after that time. Bacterial counts in the first stage and control chamber remained at about $4 \times 10^6$/ml throughout the experiment (Fig. 8). The counts in the first stage growth vessel were consistently about $0.5 \times 10^6$/ml higher than in the second stage control chamber (Fig. 8). Ciliate counts increased steadily until day four, and then oscillated about a mean of about $10^3$/ml. Such oscillations have been seen by others and are thought to possibly be due to normal predator/prey interactions.

The equations governing the dynamics of the bacterial populations in the second stage chambers are as follows:

$$dN/dt = \mu (N_o - N) - (g + g') N \qquad (4)$$

$$dN'/dt = \mu (N_o - N') - g'N' \qquad (5)$$

Where N, $N_o$, and N' are the concentrations of bacteria in the ciliate chamber, first-stage growth vessel, and control second-stage vessel, respectively, $\mu$ is the dilution rate of the second-stage vessels, g is the ciliate grazing rate in units of inverse time, and g' is the apparent loss rate of bacteria in the control vessel. In the steady state both Eqs. 4 and 5 are zero, and

$$g = \mu N_o \left(\frac{1}{N} - \frac{1}{N'}\right) \qquad (6)$$

Eq. 6 gives g in units of inverse time. In order to calculate grazing rates in terms of bacteria consumed per ciliate per unit time, the value of g was multiplied by N and divided by the concentration of ciliates. These calculations were performed on each of days 4 - 5 and 7 - 10 of the continuous culture experiment. Day 6 was excluded because of the anomalously low ciliate counts on that day (Fig. 9). On day 10 a grazing experiment was performed using microspheres. The grazing rates calculated from the continuous culture data and the microsphere experiment are listed in Table 13. A t-test showed no significant difference ($p > 0.1$) between the microsphere results from the 15 and 30 min. incubations, and no significant difference ($p > 0.1$) between the continuous culture grazing rates and either of the microsphere grazing rates.

## Discussion

As is the case with many scientific methods, there are assumptions involved which must be stated clearly and carefully tested. Only after this testing is done is it possible to

proceed with experiments to gather accurate, meaningful data. The objectives of this study were to critically examine the assumptions underlying the use of microspheres to estimate protozoan grazing rates.

First of all, the results indicated that if microspheres are treated with the protein BSA and made up in a FSW standard just prior to use, it can be assumed that the tracer amount of microspheres used remains dispersed in a uniform manner. Second, our results show no evidence that the ciliate Cyclidium discriminates between bacteria and microspheres. The data in Table 12, while consistent with the non-discrimination hypothesis, are less convincing than the results in Table 13. As already noted, the scatter in the Table 12 data precludes detecting a statistically significant grazing rate difference less than 35 bacteria per ciliate-hour, a figure almost equal in magnitude to the observed grazing rates. Furthermore, the fact that the calculated grazing rate is insensitive to bacteria/microsphere ratios which differ by a factor of only two does not prove that there is no discrimination. The degree of discrimination may have been quite large but similar in both cases. A more thorough examination of the discrimination question using this technique would require conducting experiments over a wide range of bacteria/microsphere ratios.

The continuous culture experiments provided a more rigorous test of the non-discrimination question, because the true grazing rates could be calculated from Eq. 6. However, the scatter in the data is sufficient to make detection of a difference in

calculated grazing rates smaller than 28 bacteria per ciliate-hour impossible at p = 0.05. Hence the very close agreement between the mean grazing rates calculated from Eq. 6 and from the microsphere method is fortuitous. There is probably a legitimate question as to how much the scatter in the grazing rates calculated from Eq. 6 reflects analytical noise versus true temporal variations in grazing rates. Unfortunately the microsphere method was employed only on day 10, and it was therefore impossible to examine the temporal variation in microsphere-calculated grazing rates or the correlation between the Eq. 6 grazing rates and the microsphere-calculated grazing rates. It is noteworthy however that on day 10 the discrepancy between the Eq. 6 grazing rate and the microsphere-calculated grazing rate (47 versus about 66 bacteria per ciliate-hour) was much greater than the discrepancy between the mean Eq. 6 grazing rate (71 bacteria per ciliate-hour) and the microsphere-calculated grazing rate. Based on the data in Tables 12 - 13, one might be inclined to conclude that the microsphere method yields more precise grazing rate estimates than the continuous culture method, but as already noted the continuous culture results may reflect in part true temporal variability in addition to the usual experimental error. The microsphere results in Table 13 do indicate that at the bacterial concentrations employed in this experiment an incubation time of even as much as 30 minutes does not produce a noticeable bias due to egestion of microspheres, because the grazing rates calculated from the 15 minute and 30 minute incubations differ by less than 4%. One

assumption obviously not addressed in this study is that in field work all protozoans ingest microspheres, and that the rate of ingestion is directly proportional to the microsphere/bacteria concentration ratio. This assumption can probably be addressed in a satisfactory manner by conducting experiments with a reasonably large number and variety of protozoan grazers in the laboratory. The purpose of this study was simply to get a start in troubleshooting the microsphere method, and to within the precision of the experimental data there appears to be no reason to doubt that the technique works for Cyclidium. Development of this method to accurately measure protozoan grazing rates on bacteria will help in quantitatively assessing the role of protozoans as intermediate links in the trophic food web. Other data currently lacking in the literature are measurements such as biomass production of protozoan assemblages in natural systems. This measurement is pertinent to the subject of heterotrophic flagellates which are more likely than ciliates to be the organisms controlling picoplankton populations through grazing (Sherr and Sherr, 1984). Once such measurements as grazing rate and biomass of these organisms can be made reliably it will be possible to obtain a more accurate picture of the energy and material flow in the ocean environment.

In situ Incubations with $^{14}C$-Labeled Bacteria in Kaneohe Bay. (Cynthia Tynan, with some help in data analysis from E. Laws).

## Introduction

The importance of heterotrophic protozoa in regulating bacterial concentrations has been suggested (Fenchel, 1982a,c; Taylor, 1982; Linley et al., 1983). Despite the recent interest in the role of bacteria in the cycling of dissolved organic matter (Williams, 1981; Hagstrom et al., 1979; Azam and Hodson, 1977; Linley et al., 1983) and the microbial loop (Azam et al., 1983), few in situ measurement of grazing rates by 1 - 12 μm size predators have been attempted.

There is presently a suite of methods available for the determination of bacterial growth: frequency of dividing cells; $^{35}S$-sulfate assimilation; $^{3}H$-adenine incorporation into DNA and RNA; increase in cell number; and increase in ATP (Azam and Fuhrman, 1984). An array of methods for the determination of grazing rates on bacteria also exists; radiolabeling (Hollibaugh et al., 1980; Taylor and Sullivan, 1984); inhibition (Fuhrman and McManus, 1984); dilution (Landry and Hassett, 1982; filtration (Wright and Coffin, 1984); and the use of microspheres (see Bailiff manuscript). The majority of grazing rates have been obtained using laboratory cultures of ciliates or microflagellates. The number of field measurements of grazing rates by heterotrophic protozoa is limited (Sherr and Sherr, 1984).

The present study may be one of the first attempts to obtain in situ rates of grazing by heterotrophic protozoa. Bacterial assemblages from Kaneohe Bay, grown on evenly labeled $^{14}$C-glucose were introduced into in situ grazing chambers. This method of labeling was selected over a direct introduction of isotope to the chamber (Roman and Rublee, 1981). Although the latter method eliminates the problem of alteration of cell concentrations, it does not provide for the assessment of partitioning of the label by the plankton during initial incorporation.

## Materials and Methods

Assemblages of $^{14}$C-labeled Kaneohe Bay bacteria were obtained by inoculating 100 ml of sterile low organiclabeling media (LM) (Taylor and Sullivan, 1984) with 100 µl of .8 µm Nuclepore-filtered Kaneohe Bay water. Uniformly labeled $^{14}$C-glucose, obtained from ICN Radiochemicals, was added to the incubation flask to give a final concentration of .12 µCi per ml - .43 µCi per ml. The flask was incubated in the dark on a New Brunswick G24 Environmental Incubator Shaker table at 200 rpm. The uptake of isotope by bacteria was monitored over the two day incubation phase. Subsamples of 100 µl were filtered through Whatman GF/F Glass microfiber filters. Filters were placed in film tubes with 4 ml of Aquasol-2 liquid scintillation cocktail, heat sealed and placed in plastic vials. All samples were assessed for radioactivity on a Packard Tri-Carb model 4640 liquid scintillation counter. The incubation phase was terminated when counts (dpm), reflecting the incorporation of isotope into particulates, plateaued.

A chase phase with unlabeled glucose followed the incubation phase. To replace soluble pools of $^{14}C$-compounds with soluble $^{12}C$-compounds, 1.0 µM unlabeled glucose was added to the flask. If the in situ experiment was not to commence within 6 hours the flask was kept under refrigeration during the chase phase.

At the end of the chase phase bacteria were concentrated by filtering the culture medium through a .2 µm Nuclepore filter until a thin layer of fluid remained above the filter. Bacteria were resuspended with an addition of 20 ml of filtered sterile seawater. The process of filtration and resuspension was repeated three times. The final 20 ml suspension was filtered through a 1 µm Nuclepore filter to eliminate large cells and aggregates. In the first experiment bacteria were not prefiltered through a 1 µm Nuclepore filter, and flocs may have contributed to an array of labeled size classes of particles in that experiment. The 20 ml resuspension of cells was placed in two 10 ml polystyrene tubes and later transferred to syringes for injection in grazing chambers.

During the second experiment the culture flask was very turbid at the time of harvesting. Filtration of the suspension was slow and it was necessary to collect cells by centrifugation at 10000 rpm at 20°C. Cells did not pellet well however, and the previous method of filtration and resuspension was resumed.

## In Situ Incubations

ISIS chambers (Gunderson 1973) were used for two in situ grazing experiments in Kaneohe Bay on 27 July and 10 August 1985. The volume of the clear Plexiglas chambers was 860 ml. Two chambers, an experimental and control, were deployed 1 m below the surface in the lagoon at Coconut Island. Chambers were retrieved and C-clamps were fitted over the chambers to insure a tight seal. Water from the control chamber was collected and filtered through a 1 µm Nuclepore filter. The chamber was rinsed with filtered seawater (GF/F) and the 1 µm filtrate was replaced. Labeled bacteria were injected into the chambers with a syringe, and the chambers were again lowered and gently agitated. The total activity in each chamber was determined from 1 ml subsamples. Samples were immediately drawn for analysis of particulate $^{14}C$, dissolved organic carbon (DOC) and, in the second experiments, $^{14}C$-respiration. A total of 120 ml was removed from each chamber. To determine total bacterial counts, 10 ml samples were collected from each chamber and preserved with formaldehyde at a final concentration of 2%.

In situ incubations were conducted for six hours (15:15 - 21:15) and twenty-four hours (09:30 - 09:30) in the first and second experiments, respectively. At the end of the incubations subsamples were collected for final $^{14}C$-particulate, DOC, and $CO_2$ determinations. In addition to the samples collected for bacterial enumeration, 100 ml from each chamber were preserved with Lugol's solution for total counts of microflagellates.

## Data Analysis

### $^{14}C$ Particulate

In the first experiment, two 50 ml subsamples from each chamber were filtered under low vacuum (5 mm Hg) through a series of Nuclepore filters: 12 µm, 5 µm, 3 µm, 1 µm, and .2 µm. The filtration of 50 ml through a .2 µm Nuclepore filter proved too slow, and subsequent to the first control filtration only 25 ml subsamples were used for the final filtration. During the second experiment two 25 ml subsamples were similarly processed, except for the 1 µm filtrate, which was passed through a .2 µm Nuclepore and a Whatman GF/F filter to insure that all particulate material was collected.

### $^{14}C$ Dissolved Organic Carbon (DOC)

From the .2 µm filtrate three 5 ml replicate subsamples were placed in polystyrene tubes and acidified to a pH of 2.8 with addition of .1 N HCl. Samples were frozen until they could be processed at another laboratory. After thawing, samples were sparged with air for 20 minutes under a fume hood. One ml aliquots were transfered to plastic tubes, which were heat sealed and processed in the aforementioned manner.

### $^{14}C$ Respiration

To measure $^{14}CO_2$, fluted strips of Whatman qualitative filter paper were soaked in P-phenethylamine and suspended from stoppers in serum bottles immediately prior to addition of sample. Three

replicate subsamples of 20 ml were transfered to serum bottles containing the treated wicks, and adjusted to a pH of 2.8 with sufficient .1 N HCl. Samples were stored in a cool, dry location until the remainder of the analysis could be performed. To resume analysis, serum bottles were agitated on a New Brunswick G24 environmental incubator shaker table at 200 rpm at 20°C for 40 minutes. The filter papers containing absorbed $^{14}CO_2$ were processed in the same manner as filters containing particulate material.

Bacterial and Microflagellate Counts

Bacteria were enumerated by the acridine orange direct count (AODC) method (Hobbie et al., 1977). Following the concentration and resuspension of the labeled bacterial cells, a .2 ml aliquot was removed from each polystyrene holding tube and measured by AODC. Samples for total bacterial concentration were collected from the control and experimental chambers after the addition of isotope. Samples were drawn after the chambers had been lowered and allowed to mix with gentle agitation. At the end of the incubation period samples were again collected.

To determine the concentration of microflagellates, samples were collected and preserved with Lugol's solution at the beginning and end of the incubation period. A 10 ml subsample from the experimental chamber was added to a settling chamber and allowed to settle for 24 hours; a 25 ml subsample was similarly processed for the control. Counts were reported as number per transect for two size fractions: 5 - 12 µm and 3 - 5 µm. This method was

not sensitive enough for enumeration of microflagellates less than 3 µm.

Respiration measured at the end of the incubation in the experimental chamber included bacterial as well as grazer respiration. Subtracting $^{14}CO_2$ evolved in the control (1 µm filtered) from the experimental chamber provided an estimate of grazer respiration. Similarly the release of DOC was obtained by subtracting the net release in the control from the experimental chamber. The dpms for samples of $CO_2$ and DOC were corrected to adjust for the difference in the percentage of labeled cells in the experimental and control chamber.

## Ingestion Rate

Ingestion rates were determined from the equestions of Taylor and Sullivan (1984).

Id = total ingested dpm

I = Id/S x X = ingestion rate (bacterium per liter-hour)

S = specific activity (dpm per bacterium)

X = incubation time

# Results

## Experiment 1

The results of the first in situ grazing experiment are presented for heuristic purposes. The partitioning of total isotope during

the six hour incubation is presented in Figure 10. The presence of isotope in the 3 - 12 μm size fractions at time zero may reflect the distribution of larger aggregates of labeled bacteria in the unfiltered innoculum. Bacterial concentrations remained constant in the experimental chamber and increased in the control chamber during the incubation (Figure 11). Observation of settling chambers indicated the presence of microflagellates in the control. The concentration of 3 - 5 μm and 5 - 12 μm microflagellates in the control chamber reported as number per transect, was 7 and 4 percent of the experimental values respectively (Table 14). The potential grazing pressure was therefore much lower in the control chamber, although total counts of all size fractions of microflagellates were not made. Ciliates were not observed in the settling chambers. The increase in cell number in the control chamber under low grazing pressure is equivalent to a production rate of $3.56 \times 10^6$ cells $ml^{-1}$ $day^{-1}$.

Ingestion of free bacteria by 1 - 12 μm grazers and the fragmentation and subsequent redistribution of labeled flocs by grazers could both be reflected in the isotope partitioning. During the incubation there was a decrease in the percent isotope in the >12 and 5 - 12 μm size fractions in the experimental chambers. Both chambers showed a loss of 5 - 6 percent of total isotope in the .2 - 1 μm size fraction, an increase in the percentage labeling of DOC and the evolution of labeled $CO_2$. Measurements of $^{14}C$ in the $CO_2$ fraction were not taken at time zero; however, label in the $CO_2$ accounted for 5 - 6 percent of

the total isotope at the end of the incubation. Interpretation of the results of the first experiment is difficult due to the possible presence of large aggregates of labeled cells. However, both chambers show a relatively small decrease in the percentage of label in the .2 - 1 μm size fraction.

Experiment 2

Results of the first experiment led to the following modifications for the second in situ incubation: 1) a longer incubation time (24 hours); 2) use of both GF/F and .2 μm Nuclepore filters for the final filtration to insure entrapment of all particulates and to avoid overestimation of DOC; 3) 1 μm prefiltering of the labeled innoculum; 4) rinsing of the control chamber with filtered seawater (GF/F) before addition of the 1 μm filtrate; 5) measurement of $^{14}C$ activity in the $CO_2$ at time zero; 6) measurement of bacterial concentrations in the labeled innoculum. Partitioning of the isotope in the experimental and control chambers during the second experiment is shown in Table 15. Analysis of the results presented quite a challenge for several reasons. First, the sum of the activities in the various fractions in Table 15 average only 75% of the total activity. The cause of this discrepancy in unclear. The lowest counting rates were obtained in the DOC fractions, where only 1.0 ml was counted. Counting rates in that fraction therefore ranged from 10 to 65 dpm. The results of the $^{14}C$ experiments directed by Dr. Taguchi indicated that DOC $^{14}C$ activities can be very misleading.

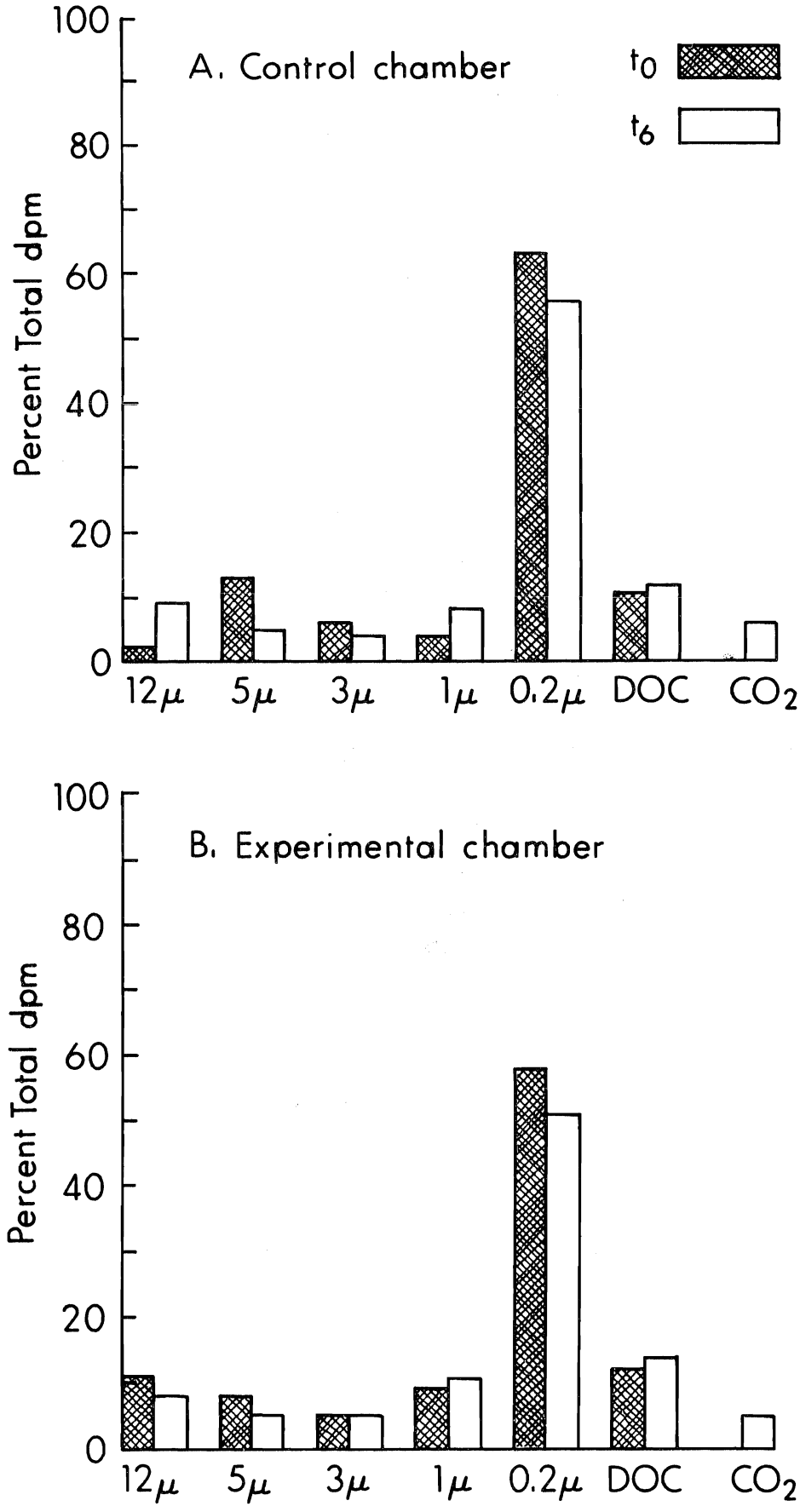



Fig. 10. Partitioning of isotope in experiment 1.

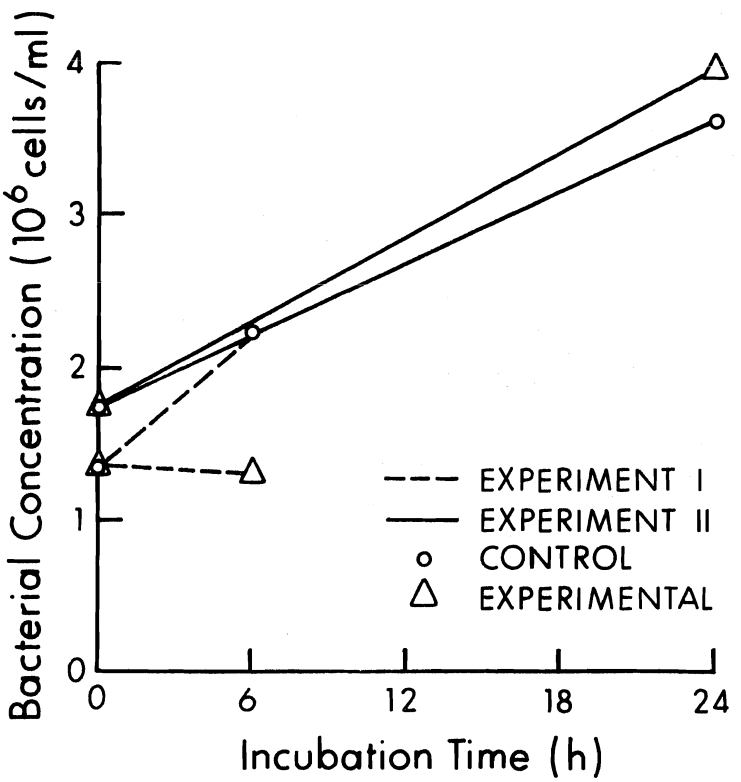



Fig. 11. Bacterial concentrations versus time in control and experimental incubators during experiments 1 and 2.

Table 14.

Mean Number of Microflagellates per transect of Settling Chamber

| Size ( µm) | experimental | control |
|---|---|---|
| 3 - 5 | 470 | 35 |
| 5 - 12 | 145 | 6 |

Table 15. Labeled bacteria grazing experiment results reported as DPM per 25 ml.

Control

| | Time = 0 | %Total | Time = 24 | % Total |
|---|---|---|---|---|
| Total | 31,216.0 | | | |
| 12 μm | 57.37 | .2 | 160.93 | .6 |
| 5 - 12 μm | 165.90 | .6 | 292.41 | 1 |
| 3 - 5 μm | 319.62 | 1 | 656.23 | 2 |
| 1 - 3 μm | 4,225.51 | 14 | 2,968.42 | 11 |
| 0.2 - 1 μm | 15,328.5 | 49 | 13,618.9 | 50 |
| DOC | 259.8 | .8 | 305.95 | 1 |
| $CO_2$ | 2,218.45 | 7 | 3,527.49 | 13 |

Experimental

| | | | | |
|---|---|---|---|---|
| Total | 127,253.0 | | 127,473.5 | |
| < 12 μm | 335.95 | .3 | 164.96 | .1 |
| 5 - 12 μm | 809.54 | .7 | 1,170.75 | .9 |
| 3 - 5 μm | 1,689.45 | 1 | 2,578.88 | 2 |
| 1 - 3 μm | 17,408.6 | 14 | 19,917.8 | 16 |
| 0.2 - 1 μm | 75,878.63 | 60 | 48,251. | 38 |
| DOC | 676.2 | .5 | 1,602.5 | 1 |
| $CO_2$ | 4,571.18 | 4 | 13,795.4 | 11 |

Second, the sum of the counts in the various fractions was quite different in the experimental chamber at the beginning and end of the incubation. Again the cause of the discrepancy is unclear. One can obviously calculate any number of ingestion rates, depending on which numbers one chooses to believe or not believe. We adopted the following procedure. First, we chose to believe the total count numbers in Table 15. For the control chamber the average total dpm were 29,163, and in the experimental chamber 127,363. We then scaled up all the counts to agree with those totals. In effect this correction amounts to assuming that all the fractionated counts are low by the same factor. The results of this exercise yield the activities listed in Table 16.

Second, it is obvious that the bacteria were not confined to the 0.2 - 1 μm fraction. In the control experiment all of the particulate counts should have been bacteria, and all the particulate counts in the experimental chamber should have been bacteria at time 0. The ratio of counts in the 0.2 - 1 μm fraction to counts in the > 1 μm fraction in these three cases is rather constant and averages 3.40 ± 0.26. Applying this ratio to the activity of 40,248 in the experimental 0.2 - 1 μm fraction at 24h, we estimate that 20,661 of the counts in the > 1 μm fraction at 24h were in fact bacteria. Hence the activity in the zooplankton grazers and their particulate waste products at 24h is estimated to be 34,697 - 20,661 = 14,036.

In the control experiment, the sum of the activities in the DOC and $CO_2$ fractions increased by 1990 dpm in 24h, which is 7.7% of

Table 16. Labeled bacteria grazing experiment results adjusted to agree with total counts. Results are dpm per 25 ml.

| Control | total dpm 29,163 | |
|---|---|---|
| | Time | |
| | 0 | 24h |
| > 12 µm | 74 | 218 |
| 5 - 12 µm | 214 | 396 |
| 3 - 5 µm | 413 | 889 |
| 1 - 3 µm | 5,459 | 4,021 |
| ( > 1 µm) | (6,160) | (5,524) |
| 0.2 - 1 µm) | 19,802 | 18,447 |
| (particulate) | (25,962) | (23,971) |
| DOC | 336 | 414 |
| $CO_2$ | 2,866 | 4,778 |

| Experimental | total dpm 127,363 | |
|---|---|---|
| > 12 µm | 422 | 240 |
| 5 - 12 µm | 1,017 | 1,704 |
| 3 - 5 µm | 2,123 | 3,755 |
| 1 - 3 µm | 21,873 | 28,998 |
| ( >1 µm) | (25,435) | (34,697) |
| 0.2 - 1 µm | 95,336 | 70,248 |
| (particulate) | (120,771) | (104,945) |
| DOC | 850 | 2,333 |
| $CO_2$ | 5,743 | 20,085 |

the activity in the bacteria at time 0. We will assume that a similar percentage of the bacterial activity at time 0 in the experimental vessel was in fact converted to DOC plus $CO_2$ by the bacteria during the 24h incubation. This conversion amounts to an increase of 9,257 dpm in the DOC plus $CO_2$ fraction. The observed increase was 15,825. Hence the metabolism of bacteria consumed by the grazers was assumed to account for an increase of 15,825 - 9,257 = 6,568 in the DOC plus $CO_2$ fraction. Using the terminology of Taylor and Sullivan (1984), we conclude that $I_d$, the total dpm ingested by grazers, was 14,036 + 6,568 = 20,604 dpm per 25 ml in the experimental chamber.

It is apparent from Fig. 11 and Table 16 that the specific activity of the bacteria in the experimental vessel decreased during the course of the incubation. At time 0 the specific activity of the bacteria was $(120,771)/[(25)\ (1.8 \times 10^6)] = 2.68 \times 10^{-3}$ dpm/bacterium, and at 24h $(70,248 + 20,661)/[\ 25)(3.96 \times 10^6)] = 9.18 \times 10^{-4}$ dpm/bacterium. This decrease in specific activity undoubtedly reflects the fact that the unlabeled bacteria were actively growing and multiplying, while the labeled cells may have been largely senescent (recall that the labeling process was terminated when particulate dpm had plateaued in the cultures). If the unlabeled cells were growing exponentially, then the specific activity would have declined exponentially, and the average specific activity of the bacteria during the course of the incubation would be $S_0(1 - S_{24}/So)\ln (So/S_{24})$, where $S_o$ and $S_{24}$ are the specific activities of the bacteria at time 0 and 24h, respectively. Since $S_o/S_{24} = 2.68 \times 10^{-3}/9.18 \times 10^{-4} = 2.92$, we

estimate the average specific activity to be $S_o(1 - 1/2.92)\ln(2.92) = 0.705S_o = 1.89 \times 10^{-3}$ dpm/bacterium. The ingestion rate is therefore estimated to be $(20604)/[(25)(1.89\times10^{-3})] = 4.4\times10^5$ bacteria $ml^{-1}d^{-1}$.

## Discussion

Few measurements of in situ grazing rates on labeled bacteria are available for comparison. Wright and Coffin (1984) obtained grazing rates of $7.5\times10^6$ bacteria per ml-day and $5\times10^6$ bacteria per ml-day for estuarine and coastal waters, respectively, of Massachusetts. These rates, obtained with filtration techniques, were computed for field samples transfered to plastic bottles and maintained in the laboratory. Microorganisms passing through a 3 µm filter were reported to exert the greatest grazing pressure on bacteria. The rates reported for coastal waters are an order of magnitude higher than the single value calculated from the second incubation in Kaneohe Bay. The importance of protozoa as grazers of bacteria may be greater for eutrophic waters (Sherr and Sherr, 1984). Possible explanations for the low grazing pressure observed in the in situ chambers include 1) paucity of grazers in the chamber or high ratio of bacteria to grazers; 2) inhibition of grazing in the chamber, i.e. bottle effects; 3) cell rupture and fragmentation upon filtration.

The low grazing rates may reflect a low concentration of grazers in the chambers. Volumetric estimates of potential grazers were not determined. The enumeration of microflagellates, likely

candidates for grazing, does not in itself generate an estimate of grazers. Microautoradiography would provide evidence of ingestion of labeled bacteria. The ratio of bacteria to grazers would have been altered by the high pulse of injected cells, 6.68 x $10^8$, in the experimental chamber.

The elegance of in situ incubations lies in the attempt to minimize environemental perturbations to the sample which might occur during transportation or maintenance in the laboratory. There is always the possibility of inhibition of grazing due to leaching from a component of the chamber. Chambers were aged in seawater prior to use and red rubber O-rings were selected over Buna-N; the latter is considered toxic to plankton (Azam, personal communication). A small amount of silicon grease was applied to the o-rings prior to deployment. It appears unlikely that grazing was inhibited in the ISIS chambers.

Filtration of cells under high vacuum and post-filtration exposure to air under vacuum can cause cell breakage and accumulation of isotope in the filtrate (Goldman and Dennett, 1985). Subsamples were filtered under low vacuum pressure, < 25 mm Hg, and filters were removed immediately upon drying. Significant cell lysis would be expected to appear in the DOC sample drawn from the .2 μm filtrate. The latter was very small in both the experimental and control samples.

Although it is possible that microzooplankton contributed to grazing pressure in the chamber, > 64 μm zooplankton have been reported to have little effect on populations of free-living and

attached bacteria (Roman, 1984). At the opposite size spectrum, it has been suggested that small eukaryotes, < .6 μm, may be responsible for a large fraction of bacterial grazing (Fuhrman and McManus, 1984). However, experiments which employ eukaryotic inhibitors, re cycloheximide, are under scrutiny (Taylor, in press; Cynar, unpublished data).

Additional measurements of in situ grazing by heterotrophic protozoa are needed to confirm or challenge the rate calculated in Kaneohe Bay. It appears from the increase in bacterial concentrations in the experimental chamber and the low percentage of incorporated isotope in the > 1 μm size fractions, that the 1 - 12 μm grazers are exerting low grazing pressure on bacteria. If grazing is not responsible for maintaining steady state concentrations of bacteria, other factors should be considered, such as availability of dissolved organic carbon and nutrient requirements in oligotrophic waters.

There was little accumulation of labeled DOC over the course of the incubations. Bacteria in the chambers may have been utilizing DOC as rapidly as it was generated. Azam et al. (1983) have advocated that tight metabolic coupling exists between DOC and bacterial uptake. To estimate bacterial incorporation of labeled DOC unlabeled bacteria would have to be added to experimental .2 μm filtrates. Bacterial incorporation of labeled DOC observed by Taylor and Sullivan (1984) was< 1.8 percent of total $^{14}C$ per hour during 2.75 - 6.0 hour incubations.

It has been estimated that 20 - 60% of primary production,

representing losses to dissolved components, enters the microbial food chain (Linly et al. 1983). However, Ducklow et al. (1986) have questioned whether this microbial loop is of much importance to higher trophic levels. They discovered that, "Only 2 percent of the label initially fixed from carbon-14-labeled glucose by bacteria was present in larger organisms after 13 days." They concluded that, "Secondary (and, by implication, primary) production by organisms smaller than 1 micrometer may not be an important food source in marine food chains. Bacterioplankton can be a sink for carbon in planktonic food webs and may serve principally as agents of nutrient regeneration rather than as food." Our results indicate that (100) (6568)/(20,604) = 32% of the bacterial carbon consumed by grazers in experiment 2 was either respired or excreted within 24h. A direct comparison with the work of Ducklow et al. (1986) is not possible, because both the time frame and complexity of the experimental systems differ greatly. However, it is obvious that even if the assimilation efficiencies of the organisms in the microbial loop were as high as approximately 70%, as our results suggest, much of the bacterial production initially available would be lost if it were necessary for the carbon to pass through more than a few transfers before reaching the metazoans and nekton.

In summary, the use of labeled bacteria to study bacteriovore grazing rates appears promising, but is not without problems. One obvious problem is the fact that it is difficult to separate the bacteria from their predators. Although most of the bacteria appear in the 0.2 - 1 μm fraction, about 30% are trapped in the

larger size categories. In our case an additional problem was created by the fact that the sum of the counts in the various size categories equaled only about 75% of the total counts. This second problem should hopefully not be a characteristic of the method and can probably be overcome with careful analytical work. The grazing rates we calculated were about 10 times smaller than rates reported from some other coastal and estuarine systems, but the rapid increase in our bacterial populations indicates that grazing was not balancing bacterial growth, and hence there is no reason to doubt the accuracy of the calculated grazing rate. However, our results do raise some questions about the factors which actually control bacterial abundances in Kaneohe Bay.

Photosynthesis Versus Irradiance in a Tryblionellae Nitzschia Species (Bacillariophyceae) (Paul Zimba).

## Introduction

Physiological adaptation to low irradiances in both planktonic and benthic diatoms is frequently observed in field samples prior to one cell division (Admiraal, 1984; Falkowski, 1980). The presence of more than one carbon assimilation pathway, or mixotrophy, is well documented in temperate marine and freshwater planktonic and benthic algae (Hellebust and Lewin, 1977; Bird and Kalff, 1986). Some centric tychoplankters use photoheterotrophic carbon assimilation under suboptimal conditions (Coughlan, 1977). However, the measurement of organic substrate uptake is difficult in the field because of bacterial utilization or organic substrates.

Many researchers using clonal isolates for laboratory experimentation disregard the fact that the original isolation medium is often autotrophic. The selection of autotrophic clones as representative of the naturally-occurring population phenotype is frequent in previous work (Hellebust and Lewin, 1977 and references therein). Also, the assumptions of tracer level radioisotope experimentation are often violated through the dilution of substrate concentration by large isotope additions (Wright and Burnison, 1979).

Knowledge of tropical edaphic diatom taxonomy and physiology is negligible. Data that enumerate cell density and also measure physiological responses to various light levels and/or substrate

additions were not found by the author for any tropical benthic diatom population. Bunt et al. (1972) measured tropical benthic primary production by inorganic radioactive carbon uptake in the Caribbean Sea. Reported values ranged from 2.5 to 13.8 mg C $m^{-2}$ $h^{-1}$. This study did not report incident light levels, algal species composition or concentration. Because carbon incorporation and the rate of adaptation to changing photic regimes is species specific (Rivkin and Seliger, 1981), concurrent measurements of photosynthesis and cell division rates for individual species are necessary to differentiate between community changes and species-specific rate changes. These rates can then be related to species abundance to determine what cells are most physiologically important in terms of carbon assimilation and growth (Malone, 1971b; Rivkin and Seliger, 1981).

Conventional methods for the measurement of autotrophic sediment production have been used to measure community assimilation with no regard to taxonomic composition, physiological state, or contamination by photosynthetic bacteria (Van Raalte et al., 1974). The measurement of species-specific assimilation rates has been limited to clonal experiments (e.g. Harding et al. 1985) or single species of field phytoplankton (Rivkin and Seliger, 1981; Taguchi and Laws, 1985). The latter technique was modified for use in this study to measure the responses of tropical edaphic diatoms to inorganic and organic substrate additions as a function or irradiance. This experiment is the first use of the single species technique in an estuarine benthic environment.

## Materials and Methods

Studies were conducted in the south sector of Kaneohe Bay, Oahu, Hawaii during July 1985 (Figure 1). Kaneohe Bay experiences highly variable light levels during summer due to clouds produced by prevailing offshore winds. Circulation in Kaneohe Bay is caused by storm surge and wind-induced mixing but was of minor importance during the study period. Salinity was nearly constant at 35 - 36$^{o}$/00 during the sampling period (Taguchi, pers. comm.). One site sampled vertically for water column structure and light penetration was near an abandoned sewage outfall (Station 1, Fig. 1). The sediment is mainly calcareous, with a rich organic flocculent layer on the surface. Photosynthetically active radiation (400 - 700 nm) was measured with a Biospherical Submarine Light Meter at the surface and at a bottom depth of 7 m. The first collection (20 July) was made at 0500 h local time and the sediment irradiance was undetectable (less than 1 $\mu E\ m^{-2}s^{-1}$. The second collection was made at 1200 h local time (25 July) when the sediment irradiance was 64 $\mu E\ m^{-2}s^{-1}$. This irradiance was the highest observed during July 1985 (five observations). Samples were collected in dark bottles to prevent photo-oxidation during transport to the laboratory.

Irradiance dependent rates of photosynthesis were measured for individual species of benthic diatoms as done previously for phytoplankton (Rivkin and Seliger, 1981; Taguchi and Laws, 1985). Within 30 minutes of sediment sample collection by SCUBA diving,

a slurry was prepared using 0.45 μm filtered station water (200 - 300 ml volume) and 4 - 5 cc of surface sediment (ca. 1 cm deep). The slurry was prefiltered with 183 m screening to remove zooplankton and macro-detritus before being placed in incubation bottles. Samples were innoculated with $NaH^{14}CO_3$ (NEN- final activity ca. 0.5 μCi $ml^{-1}$) and 6 - $^{3}H$ glucose (I.C.N. - final ca. 0.5 μ Ci $ml^{-1}$, specific activity 40 Ci/mM) and incubated for 2 - 7 h under "cool-white" fluorescent illumination with neutral density screening to attenuate irradiance to desired levels. For each irradiance, 6 - 10 replicate vials containing 2 - 10 cells were isolated after isotope uptake. Cells were harvested after incubation by passage of the labeled slurry through 35 μm mesh. A dissecting microscope (Wild M-5) was used to identify species and remove any attached material from each isolated cell. This procedure typically involved three to six transfers of isolated cells to filtered station water. The Utermohl sedimentation technique (Venrick, 1978) was used to quantify sediment core algal cell densities. Bacterial contamination on each cell was determined for one sample by epi-fluorescence microscopy after acridine orange staining (Coats and Heinbokel, 1982). Species identification of cleaned diatom frustules from one isolated vial revealed the isolate to be Nitzschia graeffei Grunow, which belongs to the section Tryblionellae of the genus.

Radioactivity was measured on dual labeled samples with a Packard 5050 liquid scintillation counter using Aquasol-2 scintillation cocktail. All samples were corrected for quenching (external

standards) and background (by subtraction of blank counts). Outlier values were removed prior to data analysis using a modified running average technique (Kruskall, 1960). Inorganic carbon uptake was calculated on a per cell basis using known alkalinity values (Parsons et al., 1984). Rates of organic and inorganic isotope assimilation were also normalized and expressed as a rate process for each substrate per unit time (Wright and Burnison, 1979). This procedure was necessary due to the low concentration of glucose present in the sediment pore water ( $< 2.7 \times 10^{-4}$ M). This upper limit of glucose concentration was determined for two sediment pore water samples collected on 18 and 25 July.

## Results

Cell densities of N. graeffei averaged 5,000 cells $cm^{-2}$. These cells were easily recognized because of their thickened yellow cell wall. Contaminating species of diatoms of similar size and shape included Surirella sp. and several Navicula spp. Other abundant algae included Anabaena sp., Dictyocha fibula Ehrenberg, and Paralia sulcata (Ehrenberg) Cleve. Initially species other than N. graeffei were isolated, including Bacillaria paxillifer Gmelin and several species of Nitzschia, Pleurosigma and Gyrosigma. These species exhibited mixotrophic carbon uptake, but because of the difficulty in species recognition at 100X magnification these were not isolated routinely. Bacillaria paxillifer was easy to recognize but was not isolated routinely because of difficulty in removing adhering detritus and in

Table 8. Productivities and productivity indices (PI) calculated from the data in Table 7. Production numbers are $mgC/m^3$. PI units are $gC.g^{-1}Chl\underline{a}.h^{-1}$ during the photoperiod. Cha concentrations are $mg/m^3$.

| Time Interval (hours) | Production Size Fraction 0.2 - 2 m | 2 - 10 m | > 10 m |
|---|---|---|---|
| 0 - 6 | 16.6 | 10.2 | 4.2 |
| 6 - 12 | 4.5 | 0.4 | 3.2 |
| 0 - 12 | 21.1 | 10.6 | 7.4 |
| 0 - 24 | 21.9 | 9.6 | 9.1 |
| | Photoperiod Averages | | |
| Chl<u>a</u> | 0.133 | 0.165 | 0.038 |
| PI | 13.2 | 5.3 | 16.4 |

counting cells per colony. Bacteria adhering to N. graeffei were enumerated on 5 cells from a sediment core collected on 31 July. An average of 18 bacteria were identified on each uncleaned diatom frustule.

Maximum inorganic carbon uptake by N. graeffei was ca. 82 pg. C $cell^{-1}$ $hr^{-1}$, with saturation occurring at ca. 89 $\mu E$ $m^{-2}$ $s^{-1}$ for cells isolated from sediments illuminated by 65 $\mu E$ $m^{-2}$ $s^{-1}$ *in situ* (Figure 13). Cells isolated from samples collected prior to sunrise did not saturate autotrophically at similar and higher light levels (Figure 12).

Heterotrophic carbon assimilation was evident. However, these rates were over an order of magnitude less than autotrophic rates. Light stimulated glucose assimilation in cells adapted to the light (Figure 13) and in cells isolated from the sample collected prior to sunrise (Figure 12). The assimilation rate for glucose was nearly the same in both populations, although previously light-conditioned cells saturated at higher light levels photoheterotrophically. The presence of bacteria on the algal cells isolated would increase the apparent glucose assimilation by the algae; however, this effect would be minimal due to the difference in cell surface are if the $k_m$ values for bacteria and algae are similar (Cole, 1982).

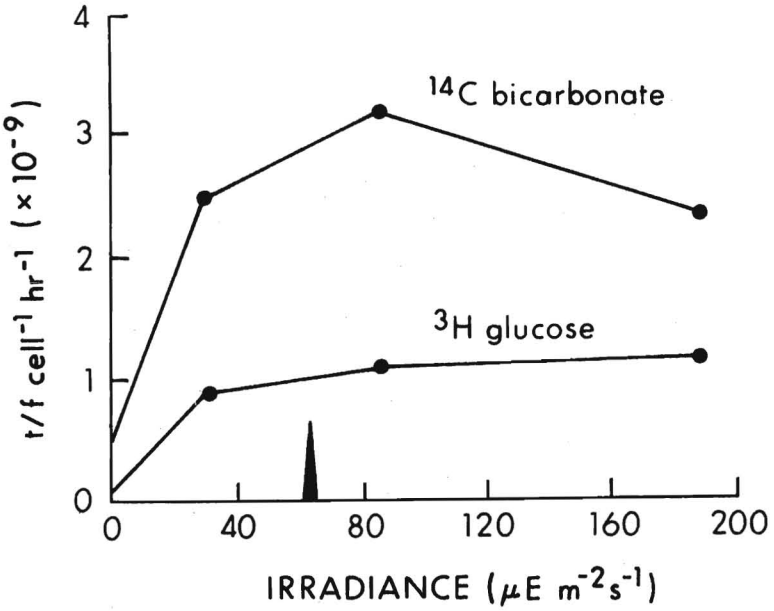



Fig. 13. Results as in Fig. 12 for experiment of July 25. Samples were collected at 1200h local time when the sediment was receiving 64 μEinst $m^{-2}s^{-1}$(arrow).

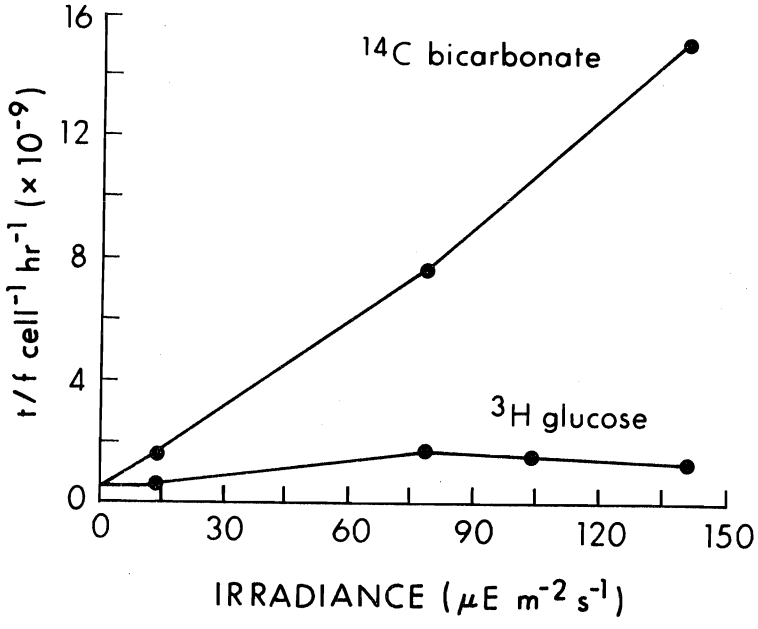



Fig. 12. Photosynthesis versus irradiance relationship for Nitzschia graeffei incubated with $^{3}$H glucose and $^{14}$C bicarbonate on 20 July computed as assimilation per cell per hour. Assimilation was calculated as the amount of label incorporated (t) as a fraction of the total available (f). Samples were collected at 0500h local time when the sediment was receiving less than 1.0 μEinst m$^{-2}$s$^{-1}$.

Sediment surface samples collected in temperate or tropical waters contain a diverse diatom species composition (Foged, 1984). Nitzschia graeffei has been reported from several tropical locations of the New World: Puerto Rico (Hagelstein, 1939), Trinidad (Boyer, 1927), and Cuba (Foged, 1984). The morphologically similar and perhaps conspecific N. jelineckii Grunow has been reported from Cuba (Foged, 1984) and Trinidad (Hagelstein, 1939). N. graeffei appears to be distributed in tropical and subtropical coastal waters.

The single species isolation technique provides the opportunity to assess algal-bacterial interactions in field samples. Bacterial abundance on N graeffei was similar to values obtained from water column species of Anabaena. An average of 11 bacterial cells were enumerated from heterocysts in a freshwater environment (Cole, 1982).

Diatoms having high surface:volume ratios isolated from temperate intertidal mudflats exhibit light stimulated organic assimilation capabilities (Admiraal, 1984). Mixotrophic growth rates of ca. 75% of the maximum were measured at irradiances between 15 - 300 $\mu E\ m^{-2}s^{-1}$. Genera such as Surirella, Pleurosigma, Navicula and Nitzschia may compete with bacteria for organic substrates when concentrations are sufficient for the stimulation of the necessary membrane transport system.

Admiraal (1984) reported that the time of incubation was critical in detecting photoinhibition responses. This concern is

warranted when considering data from 2 and 7h incubations (Zimba, unpublished). Short term incubations (2h) at high light (89 $\mu E\ m^{-2}a^{-1}$) photoinhibited inorganic carbon uptake, while longer ( > 7h) incubations at the same light intensity did not. These data suggest that cells adapted to a low light regime need several hours to modify their photosynthetic apparatus to the (new) higher irradiance conditions.

These data do not agree with the findings of Darley et al. (1979). They reported that motile algae isolated from a Georgia salt marsh were able to satisfy 1% of the cell's carbon requirement mixotrophically. Betweeen 6 - 40% of N. graeffei's carbon uptake was accomplished through photoheterotrophy. Because at least one numerically important species is able to assimilate organic carbon, any attempt at budgeting ecosystem energy flow and carbon flux should include these values. The values generated by inorganic $^{14}C$ assimilation alone would then represent underestimates of total algal benthic growth from these regions when organic substrates are present in millimolar concentrations (Bunt et al., 1972). Glucose concentrations often exceed the millimolar range in estuaries (Cooksey and Chansang, 1976). Additionally, anoxic sediments would reduce available bicarbonate concentrations due to low pH. The carbonate concentration in seawater is typically about 2mM. The ability of algae to use available alternative substrates would increase the chances for survival of these species.

## Figures

Figure 1. Sampling location (dark circle) in Kaneohe Bay, Hawaii during summer 1985.

Figure 2. Photosynthesis versus irradiance relationship for Nitzschia graeffei incubated with $^{3}H$ glucose and $^{14}C$ bicarbonate on 20 July computed on a cell $h^{-1}$ basis. Assimilation was computed as the amount of label incorporated (t) as a fraction of the total available (f). Linear regression $r^2$ values are shown for each substrate for the linear portions of each curve. Samples were collected at 0500 h local time when the sediment surface was receiving less than 1 $\mu E\ m^{-2}\ s^{-1}$.

Figure 3. Photosynthesis versus irradiance relationship for Nitzschia graeffii incubated with $^{3}H$ glucose and $^{14}C$ bicarbonate on 25 July computed on a cell $h^{-1}$ basis. Assimilation was computed as amount of label incorporated (t) as a fraction of the total available (f). Linear regression $r^2$ values are shown for each susbrate for the linear portions of each curve. Samples were collected at 1200 h local time when the sediment surface was receiving 64 $\mu E\ m^{-2}\ s^{-1}$ (arrow).

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